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RESEARCH PAPER

Evidence for the localization of the *Arabidopsis* cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum

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Abstract

Cytokinins are hormones that are involved in various processes of plant growth and development. The model of cytokinin signalling starts with hormone perception through membrane-localized histidine kinase receptors. Although the biochemical properties and functions of these receptors have been extensively studied, there is no solid proof of their subcellular localization. Here, cell biological and biochemical evidence for the localization of functional fluorophor-tagged fusions of *Arabidopsis* histidine kinase 3 (AHK3) and 4 (AHK4), members of the cytokinin receptor family, in the endoplasmic reticulum (ER) is provided. Furthermore, membrane-bound AHK3 interacts with AHK4 *in vivo*. The ER localization and putative function of cytokinin receptors from the ER have major impacts on the concept of cytokinin perception and signalling, and hormonal cross-talk in plants.

Key words: AHK3, cytokinin perception, endoplasmic reticulum, FIDSAM.

Introduction

Cytokinins, a class of adenine-derived plant hormones, have been implicated in almost every aspect of plant growth and development, including root and shoot growth, vasculature differentiation, photomorphogenesis, senescence, fertility, and seed development (Muller and Sheen, 2007*a*; Werner and Schmulling, 2009) as well as in responses to cold and osmotic stress (Tran *et al.*, 2007; Jeon *et al.*, 2010). It is well established that cytokinin perception and signalling is mediated by a multistep two-component circuitry. In *Arabidopsis thaliana* three transmembrane histidine kinases, namely AHK2, AHK3, and AHK4, serve as cytokinin receptors (Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Ueguchi *et al.*, 2001). Cytokinin binding to their CHASE domain is proposed to initiate autophosphorylation of the receptors at a conserved histidine residue in the transmitter domain (Pas *et al.*, 2004; Muller and Sheen, 2007*b*). The phosphoryl group is then transferred to a conserved aspartate residue in the receptor's receiver domain. Histidine phosphotransfer proteins (AHPs) finally transmit the signal to response regulators (ARRs), which then regulate the cellular responses (Muller and Sheen, 2007*b*; Werner and Schmulling, 2009; Kieber and Schaller, 2010).

Although the cytokinin receptors have been extensively studied regarding their specific functions, biochemical properties, and expression patterns (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006; Romanov *et al.*, 2006), their subcellular localization and molecular function are still not fully determined. It has been assumed that they reside in the plasma membrane, and a green fluorescent protein (GFP) fusion of AHK3 appears to localize to the plasma membrane of protoplasts (Kim *et al.*, 2006). However, further attempts to ascertain this localization led to the observation that they show a more diverse localization pattern (Dortay *et al.*, 2008).

In this report, cell biological and biochemical evidence for the localization of functional fluorescent protein fusions of AHK3—a representative of the cytokinin receptor family—in the endoplasmic reticulum (ER) is provided. This localization is detected not only in transiently transformed tobacco (*Nicotiana benthamiana*) and *Arabidopsis* cells but also in stably transgenic *Arabidopsis* plants. The present observation entails a reconsideration of the current model of cytokinin signal perception and, as other hormone receptors are also located in the ER, opens new perspectives for hormonal cross-talk at this cellular compartment in plant cells.

Materials and methods

Construction of cDNA fusions

To generate the fusion proteins, attB sites were added via PCR-mediated ligation to the coding regions of *AHK1* (AT2G17820), *AHK3* (AT1G27320), *AHK4* (AT2G01830), *ERS1* (AT2G40940), and *NHL3* (AT5G06320) with or without a STOP codon and recombined into pDONR™201 according to the manufacturer's protocol (Invitrogen). The cDNA was then transferred via LR reaction (Invitrogen) into the destination vectors pH7WGF2, pH7FWG2, or pB7WGR2 (Karimi *et al.*, 2002) and pABindmCherry (Bleckmann *et al.*, 2010).

For constructs under the control of the *ubiquitin 10* (*UBQ10*) promoter, a gateway cassette (reading frame A) was inserted into the vectors pUGT1kan+ and pUGT2kan+ (Karin Schumacher, unpublished) at the *Sma*I site in the multiple cloning site. The *AHK3* coding sequence was then inserted in the destination vectors by LR reaction.

For the fusion construct with internal GFP (*AHK3intGFP*), linker sequences (coding for GGGGS/T) were added via PCR to the coding sequence of GFP using the primers GFP-BcuI-S and GFP-BcuI-A (Supplementary Table S1 available at *JXB* online). For ligation into the AHK3 entry clone an appropriate restriction site was produced via site-directed mutagenesis in the AHK3 coding sequence at position 123 (corresponding to amino acid 41) where the linker–GFP–linker sequence was introduced. The *AHK3intGFP* cDNA was then recombined into pMDC32 (Curtis and Grossniklaus, 2003) by LR reaction. For mating-based split-ubiquitin system (mbSUS) assay, the *AHK4* cDNA was transferred

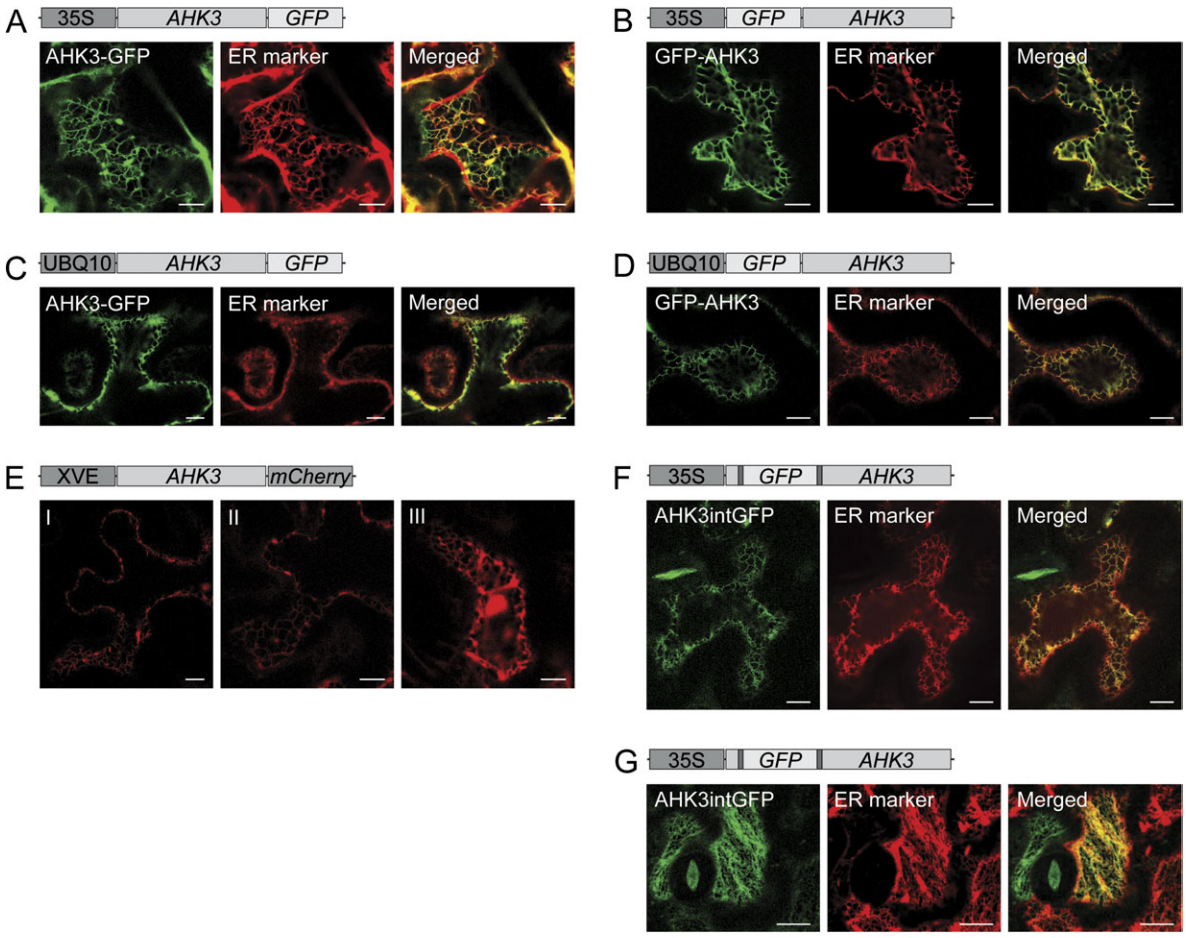

Fig. 1. The *Arabidopsis* cytokinin receptor AHK3 localizes to the ER in transiently transformed tobacco leaf cells and *Arabidopsis* seedlings. (A–D) and (F) Confocal images of transiently transformed tobacco epidermal leaf cells co-expressing the indicated AHK3 fusion protein under the control of the *35S* promoter or the *UBQ10* promoter with the ER marker ER-rk CD3-959. (E) Confocal images of transiently transformed tobacco epidermal leaf cells expressing an AHK3–mCherry fusion protein under the control of the estradiol-inducible promoter (XVE). Images were recorded 2 h (I), 4 h (II), and 24 h (III) after application of 20 μM β-estradiol. Images (I) and (II) were recorded at the highest sensitivity settings of the microscope at which the mCherry fluorescence was just detectable. (G) Confocal images of transiently transformed *Arabidopsis* cotyledon cells co-expressing the indicated AHK3 fusion protein with the ER marker ER-rk CD3-959. Bars represent 10 μm.

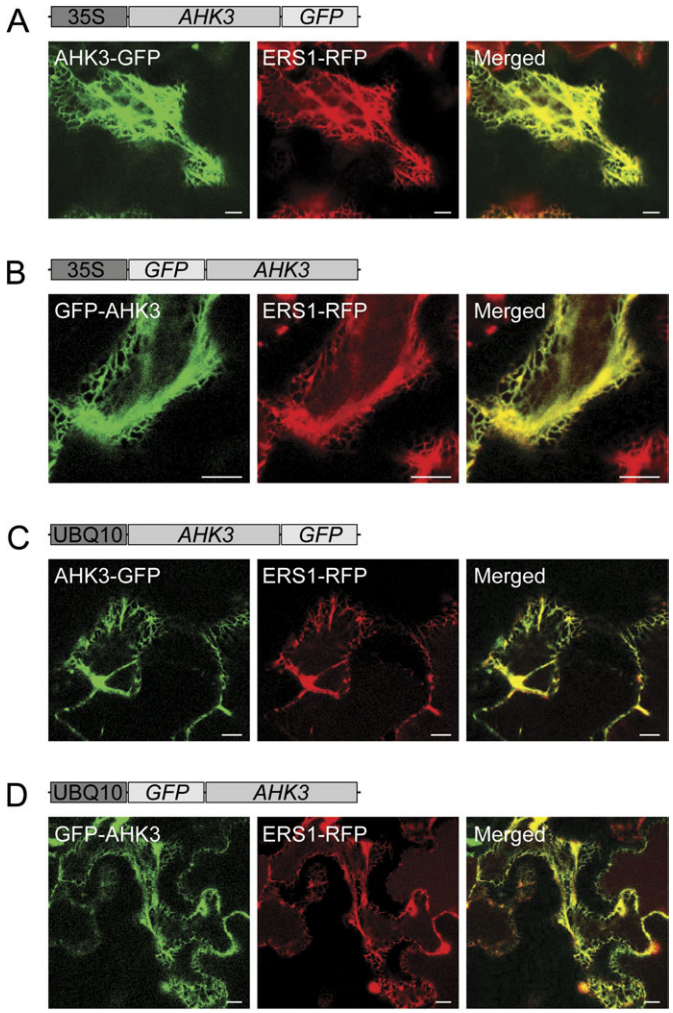

Fig. 2. AHK3–GFP and GFP–AHK3 fusion proteins co-localize with ERS1–RFP. (A–D) Confocal images of transiently transformed tobacco epidermal leaf cells co-expressing the indicated AHK3 fusion protein under the control of the *35S* or the *UBQ10* promoter and an RFP fusion of the ethylene receptor ERS1 (ERS1–RFP). Bars represent 10 μm.

by LR reaction to pMetYC-Dest and the *AHK3* and *ERS1* cDNAs to pXNubA22 (Grefen *et al.*, 2009).

Transient gene expression in Nicotiana benthamiana *leaves and* Arabidopsis *seedlings*

Transient transformations of *N. benthamiana* leaves with the *Agrobacterium tumefaciens* strain GV3101 pMP90 containing the expression constructs were carried out as described in Schutze *et al.* (2009). The transformed leaves were assayed for fluorescence by confocal laser scanning microscopy (CLSM) 2–3 d post-infiltration. The transgene expression from the estradiol-inducible promoter was induced 2–3 d after infiltration with 20 μM β-estradiol supplemented with 0.1% Tween-20. For transient expression in *Arabidopsis* seedlings, the *Agrobacterium* strains containing the fusion constructs and the marker constructs used were grown as described (Marion *et al.*, 2008), prior to infiltration diluted in 5% sucrose, 200 μM acetosyringone to an OD_{600} of 2.0, and mixed 1:1. 3–4 d old *Arabidopsis efr1* seedlings (Zipfel *et al.*, 2006) were transformed via vacuum infiltration as described by Marion *et al.* (2008) and the seedlings were examined for fluorescence 3 d post-infiltration.

CLSM and fluorescence intensity decay shape analysis microscopy (FIDSAM)

CLSM and FIDSAM as well as the used spectromicroscopic systems and measurement protocols have been described previously (Elgass *et al.*, 2009; Schleifenbaum *et al.*, 2010).

Construction of transgenic Arabidopsis *lines*

The transgenes were transformed into *Arabidopsis ahk2-2ahk3-3* plants (*ahk2ahk3*, Higuchi *et al.*, 2004) via the floral dip method and selected by phenotype (complemented dwarf phenotype of the *ahk2ahk3* mutant background). Twenty independent *ahk2-2ahk3-3* lines complemented by *AHK3-GFP* and 10 independent lines complemented by *GFP-AHK3* were isolated. After verification of the transgene integration, the lines were analysed for the GFP fluorescence signal using CLSM and FIDSAM. The line with the most intense GFP signal was used for imaging and endogylcosidase H (EndoH) assays.

EndoH assay

The EndoH assay was performed according to the manufacturer's manual (New England BioLabs) by using crude protein extracts of transiently transformed tobacco or *Arabidopsis* leaves. The proteins were analysed by SDS–PAGE and western blot using a GFP antibody.

Root growth and yeast mbSUS assays

For the root elongation assay, seedlings were grown vertically on 0.5× MS plates supplemented with different concentrations of kinetin. The root length was measured 6 d post-germination. The yeast mbSUS assays using *AHK4-Cub-PLV* and the *NubA* fusions of *AHK3* and *ERS1* as constructs were carried out as described previously (Grefen *et al.*, 2009; Caesar *et al.*, 2011).

Results and Discussion

FP fusions of AHK3 localize to the ER

In order to examine the subcellular localization of AHK3, C- and N-terminal GFP fusions of the receptor were transiently expressed in leaf epidermal cells of *N. benthamiana* and in cotyledon cells of *Arabidopsis* seedlings under the control of either the 35S *Cauliflower mosaic virus* (*35S*) or the *Arabidopsis UBQ10* promoter. Both AHK3–GFP and GFP–AHK3 showed an ER-like localization pattern in tobacco and *Arabidopsis* independent of the promoter used (Fig. 1A–D, Supplementary Fig. S1 at *JXB* online). The identity of the endomembrane system as ER was verified by the co-localization of the GFP fusions of AHK3 with the mCherry-tagged ER marker ER-rk CD3-959 (Nelson *et al.*, 2007; Fig. 1A–D, Supplementary Fig. S1B, C). In addition, there was a co-localization of the GFP fusion proteins of AHK3 with the ER-localized red fluorescent protein (RFP) fusion of the ethylene receptor ERS1 (Grefen *et al.*, 2008; Fig. 2). In contrast, no co-localization of these AHK3 fusion proteins was found with the mCherry-tagged Golgi marker G-rk CD2-967 (Nelson *et al.*, 2007; Supplementary Fig. S2) and the RFP fusion of the plasma membrane protein NHL3 (Varet *et al.*, 2003; Fig. 3). Furthermore, the GFP fusion of the plasma membrane-bound AHK1, which is a positive regulator of drought and salt stress response and functions as an osmosensor in yeast (Urao *et al.*, 1999; Tran *et al.*, 2007;

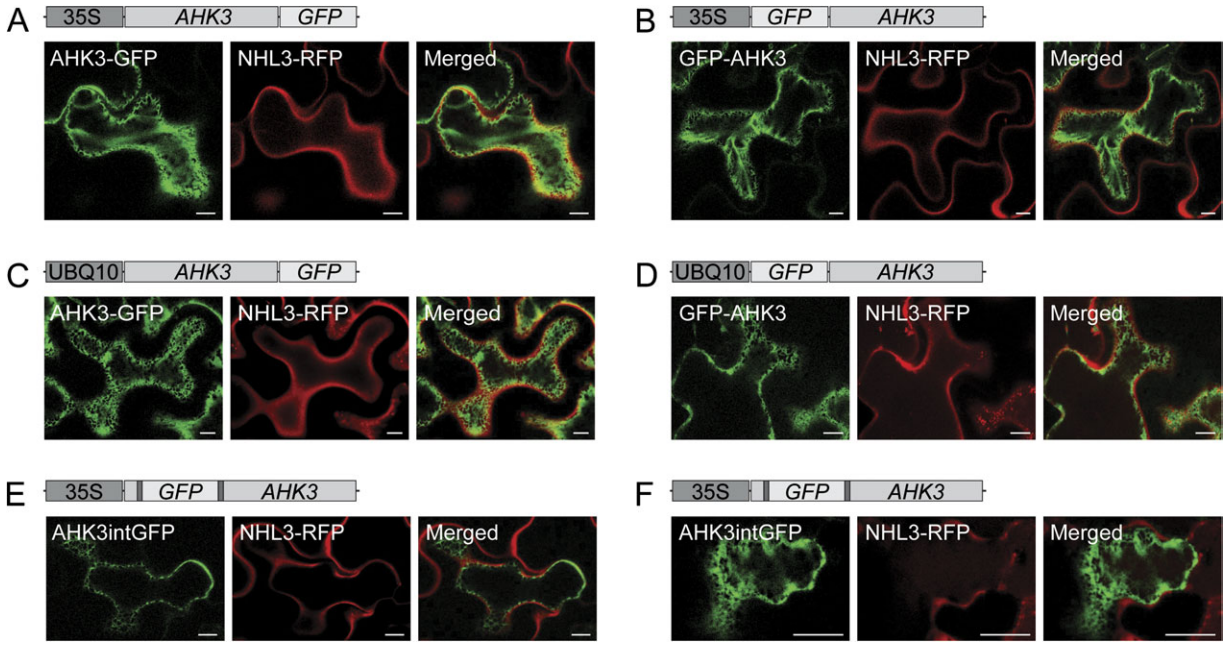

Fig. 3. AHK3–GFP and GFP–AHK3 fusion proteins do not co-localize with the plasma membrane-localized fusion protein NHL3–RFP. (A–E) Confocal images of transiently transformed tobacco epidermal leaf cells co-expressing the indicated AHK3 fusion protein under the control of the *35S* promoter or the *UBQ10* promoter and the plasma membrane-localized fusion protein NHL3–RFP. (F) Confocal images of transiently transformed *Arabidopsis* cotyledon cells co-expressing the indicated AHK3 fusion protein and the plasmalemma-localized fusion protein NHL3–RFP. Bars represent 10 μm.

Wohlbach *et al.*, 2008), also did not co-localize with an mCherry fusion of AHK3 (Fig. 4). The yellow colour, partially visible in the merged images of Supplementary Fig. S2D, and Figs 3A, C, 4, results from the very strong GFP signal in this area and the physically restricted resolution of light microscopy below Abbe's diffraction limit (250 nm). However, the magnified images of these yellow domains showed an incomplete overlap of the GFP and RFP or mCherry fluorescence (Fig. 4; Supplementary Fig. S3), indicating that the fusion proteins localize to different membrane compartments.

It has been reported that strong expression of fusion proteins under the control of promoters such as *35S* or *UBQ10* might lead to mislocalization artefacts (Bleckmann *et al.*, 2010). A C-terminal mCherry fusion of AHK3 (AHK3–mCherry) expressed from an estradiol-controlled promoter system was therefore used to trigger the expression level of the histidine kinase (Bleckmann *et al.*, 2010). After transformation of the construct into *N. benthamiana* leaves, the expression was induced by brushing the leaves with β-estradiol. AHK3–mCherry fluorescence was detectable 2 h after β-estradiol application at the earliest (Fig. 1E). Already at this early time point the AHK3–mCherry fluorescence displayed a net-like ER localization pattern, which did not change within the next 22 h (Fig. 1E). The localization of β-estradiol-induced AHK3–mCherry was never observed in the plasma membrane.

The amino acid sequence of AHK3 contains potential signals for the secretory pathway and ER export, respectively [Fig. 6A; iPsort Prediction, http://ipsort.hgc.jp/ (Bendtsen *et al.*, 2004); YLoc Prediction, www.multiloc.org/YLoc (Hanton *et al.*, 2005; Langhans *et al.*, 2008; Briesemeister *et al.*, 2010)]. To exclude a possible mislocalization of the fusion proteins due to masking of potential sorting signals, a construct was generated where GFP is inserted between the first and the second predicted transmembrane domain of AHK3 (AHK3intGFP; see Fig. 6A for the details of the insertion site). AHK3intGFP showed the identical ER localization pattern to AHK3–GFP and GFP–AHK3 when transiently expressed in tobacco leaves as well as in *Arabidopsis* seedlings, and co-localized with the mCherry-tagged ER marker (Fig. 1F, G) but not with the plasma membrane marker NHL3–RFP (Fig. 3E, F).

Signal-induced translocation of receptors to the plasma membrane is reported for animal systems (Shuster *et al.*, 1999; Song *et al.*, 2004). Therefore, assays were carried out to determine whether the application of kinetin, a synthetic cytokinin, has an influence on the subcellular localization of the N- and C-terminal GFP fusions of AHK3. However, 4 h of kinetin treatment did not alter the ER localization of AHK3–GFP and GFP–AHK3 (Supplementary Fig. S4 at *JXB* online).

Furthermore, it was tested whether the intracellular location of AHK3 changed when it was co-expressed with an RFP fusion of its sister receptor, AHK4. As shown in Fig. 5A, both fusion proteins co-localized in the ER. To determine whether AHK3 is able to interact with AHK4 in the membrane, *in vivo* interaction studies were performed using the yeast mbSUS (Grefen *et al.*, 2009). The mbSUS experiments revealed that AHK3 not only forms homo-oligomers (data not shown) but also interacts with AHK4 *in vivo* (Fig. 5B). No interaction was observed with ERS1 (Fig. 5B), which also localized to the ER (Fig. 2; Grefen *et al.*, 2008). These

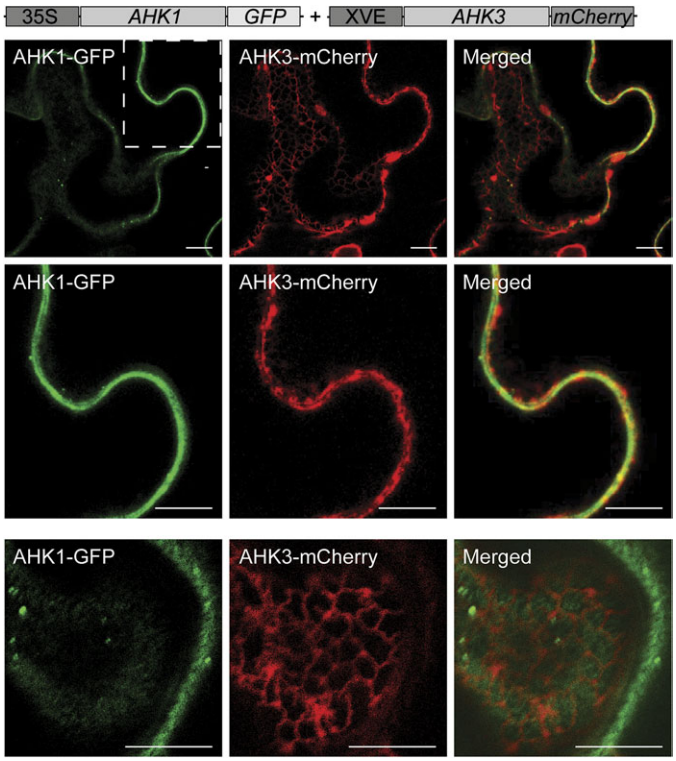

Fig. 4. AHK3–mCherry does not co-localize with a GFP fusion of the plasma membrane-bound *Arabidopsis* histidine kinase 1 (AHK1–GFP). Confocal images of different magnification of transiently transformed tobacco epidermal leaf cells co-expressing AHK3–mCherry under the control of the estradiol-inducible promoter (XVE) and AHK1–GFP under control of the *35S* promoter. The image series of the second row represents a magnified detail of the images of the first row. The image series of the third row derives from an independent cell. Images were recorded 4 h after application of 20 μM β-estradiol. Bars represent 10 μm.

data suggest that the cytokinin receptors are able specifically to homo- and heterodimerize in the ER and that their interaction has no influence on their subcellular localization.

In conclusion, all tested fluorescent protein fusions of AHK3—no matter whether GFP is tagged to the C-terminus, N-terminus, or internally—show an ER localization. A mislocalization of the fusion proteins due to overexpression or masking of potential sorting signals is unlikely. Furthermore, neither cytokinin application nor the co-expression of AHK3 and 4 or their potential *in planta* interaction are capable of altering the ER localization of AHK3.

The AHK3 protein is EndoH sensitive

To substantiate the ER localization of the AHK3 fusion proteins, a biochemical survey was conducted applying an EndoH assay. EndoH is a glycosidase which cleaves asparagine-linked oligomannose and hybrid, but not complex oligosaccharides from glycoproteins (Maley *et al.*, 1989). EndoH, therefore, enables, by electrophoretic mobility shift, the differentiation of ER-localized glycoproteins from glycoproteins in the plasma membrane, whose asparagine-linked glycans are further modified in the secretory pathway and are no longer substrates for the glycolytic enzyme (Hong *et al.*, 2008). In the AHK3 amino acid sequence, five potential N-X-S/T glycosylation sites were identified; one N-terminally of and three within the CHASE domain, and one in the receiver domain close to the C-terminus (Fig. 6A, B). Therefore, a mobility shift of EndoH-treated AHK3 would be expected on condition that AHK3 is located in the ER. As controls, AHK1–GFP, which is a plasma membrane-localized protein (Fig. 4) and has nine potential N-X-S/T glycosylation sites (Fig. 6B), and ERS1–GFP, which is, like ERS1–RFP, bound to the ER (Grefen *et al.*, 2008), were used. The ERS1 single N-X-S/T site is predicted not to be glycosylated due to its C-terminal location (Fig. 6B; Gavel and von Heijne, 1990). Total crude protein extracts of tobacco leaves expressing the GFP fusion proteins were exposed to EndoH or mock treated. After SDS–PAGE and western blot using a GFP-specific antibody, the fusion proteins were analysed for changes in their electrophoretic mobility. There was no mobility shift and, thus, no EndoH sensitivity of plasma membrane-bound AHK1–GFP or of ER-bound ERS1–GFP detected, indicating that AHK1–GFP is not retained in the ER and ERS1 is not glycosylated in tobacco cells (Fig. 6C). The unaltered pattern of AHK1–GFP in particular also proves that the reaction mixture conditions *per se* have no influence on the electrophoretic mobility of the fusion proteins. In contrast, the EndoH-treated AHK3 fusion proteins showed a significant mobility shift compared with the non-treated control (Fig. 6C). Most importantly, there was no high mobility band in the non-treated AHK3 preparations.

The results of the EndoH assays thus support the cell biological observations that the GFP fusions of AHK3 localize to the ER. Furthermore, there appears to be no subfraction of AHK3 in the plasmalemma because the entire population of the cytokinin receptor carries EndoH-sensitive mannose structures typical for ER-resident glycoproteins.

AHK3–GFP and GFP–AHK3 rescue the cytokinin-insensitive phenotype of the ahk2ahk3 *receptor mutant*

To determine the functionality of the GFP fusions of AHK3, their capability to complement the dwarf and cytokinin-insensitive root growth phenotype of the *ahk2-2ahk3-3* (*ahk2ahk3*) mutant was analysed. The *ahk2-2* or the *ahk3-3* single mutants were not used for the complementation analysis as they show the wild-type phenotype (Higuchi *et al.*, 2004). Those *ahk2ahk3* plants were selected whose dwarf and cytokinin-insensitive root growth phenotypes were complemented by *UBQ10*-driven expression of *AHK3-GFP* or *GFP-AHK3* demonstrating that both fusion proteins are functional receptors (Fig. 7A–C). Next the *AHK3-GFP*-complemented transgenic line was studied for the accumulation and subcellular localization of the fusion protein using standard CLSM. Weak fluorescence signals were detected in epidermal and stomatal cotyledon cells. The fluorescence signal appeared in a net-like and discontinuous pattern as well as in the perinuclear space (Fig. 7D). This observation suggests that AHK3–GFP is predominantly localized in the ER. To

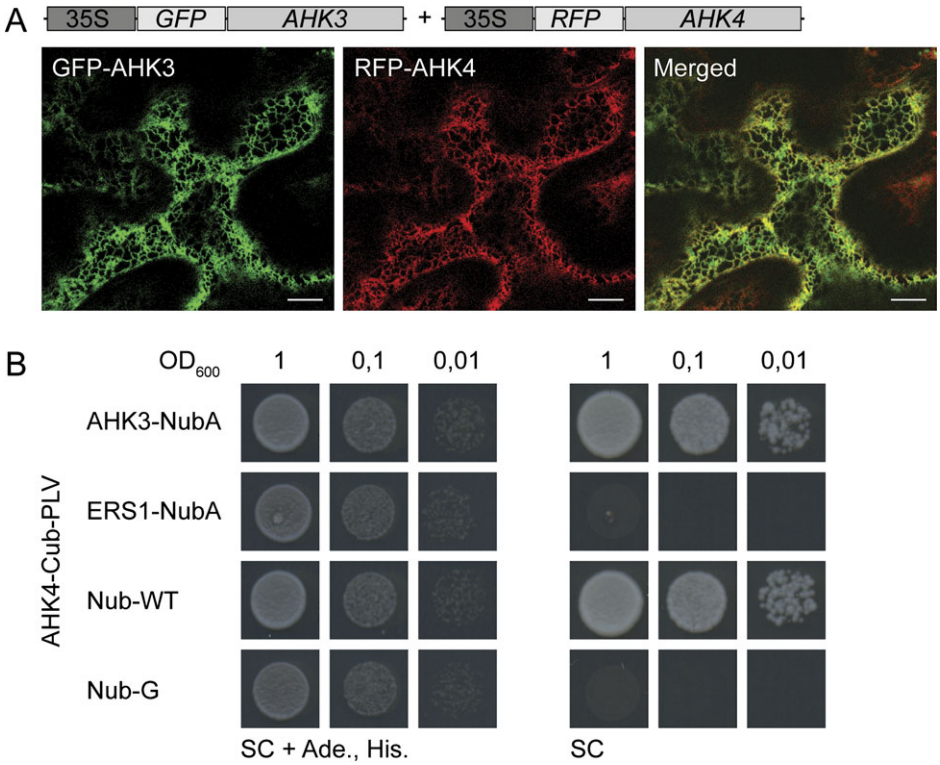

Fig. 5. The cytokinin receptor AHK4 co-localizes with AHK3 in the ER and interacts with AHK3 *in vivo* (yeast). (A) Confocal images of transiently transformed tobacco epidermal leaf cells co-expressing RFP–AHK4 and GFP–AHK3 under control of the *35S* promoter. Bars represent 10 μm. (B) Yeast mbSUS protein–protein interaction analysis. The *AHK4-Cub-PLV* construct was transformed in yeast strain THY.AP4 (MATa), and the Nub constructs of AHK3 and ERS1 were transformed in yeast strain THY.AP5 (MATα). After mating, activation of the reporter gene was determined by growth of the transformants in a dilution series (OD_{600nm} from 1 to 0.01) on SC medium (SC). The presence of the plasmid was assayed by growth on SC medium supplemented with adenine and histidine (SC+Ade., His.). Co-transformations of the AHK4–Cub-PLV fusion with NubG served as negative control and co-transformation with NubWT served as positive control.

be sure that background autofluorescence was not recorded, FIDSAM was applied. FIDSAM enhances the contrast of fluorescence images due to efficient background fluorescence repression (Schleifenbaum *et al.*, 2010). In the FIDSAM images, AHK3–GFP again became visible as a discontinuous fluorescence pattern with a net-like structure that is typical for ER-localized fusion proteins (Supplementary Fig. S5 at *JXB* online) but atypical for plasma membrane proteins (Grefen *et al.*, 2008). These data demonstrate that the observed fluorescence actually derives from the GFP of AHK3–GFP. The low accumulation of AHK3–GFP fusion protein was surprising as the *AHK3* fusion constructs were under the control of the constitutive *UBQ10* promoter, which usually provides for a high level of accumulation of the corresponding fusion protein. This suggests that the transgenic plants must keep the AHK3 protein amount at a level which is similarly low as that of wild-type *Arabidopsis*.

To substantiate the ER localization, EndoH assays were performed, using extracts from the AHK3–GFP line, the ERS1–GFP line, and the *ahk2ahk3* mutant. Again, an EndoH-caused mobility shift of the AHK3 fusion protein was observed, but not a clear shift of ERS1–GFP (Fig. 7E). Again, there was no high mobility band in the mock-treated AHK3 preparations (Fig. 7E) and no free GFP (data not shown). Thus, the observed complementation of the *ahk2ahk3* mutant phenotype was not due to post-translational cleavage of the GFP and release of non-tagged AHK3 or a translocation of an AHK3 subpopulation to the plasma membrane.

Summarizing, the results of the EndoH assay and the CLSM/FIDSAM analysis suggest that AHK3–GFP localizes to the ER not only in transiently transformed tobacco and *Arabidopsis* cells but also in transgenic plants. As AHK3–GFP complements the cytokinin-insensitive phenotype of the *ahk2ahk3* mutant and as there is no indication that a subpopulation of AHK3 targets to the plasma membrane, the receptor appears to function from the ER. However, the possibility that minuscule amounts of AHK3 are transferred to the plasma membrane, which are detectable neither by CLSM nor by western blot after EndoH treatment, cannot be entirely excluded.

Conclusion

The ER localization of AHK3 (and AHK4) has major consequences for the concept of cytokinin perception and signalling in plants. The present data indicate that the cytokinin-binding CHASE domain is not oriented to the apoplast, as previously assumed, but exposed to the ER lumen, whereas the C-terminal kinase domain, that, upon activation, transfers the phosphoryl residues to the nucleocytoplasmic histidine phosphotransfer proteins, is exposed to

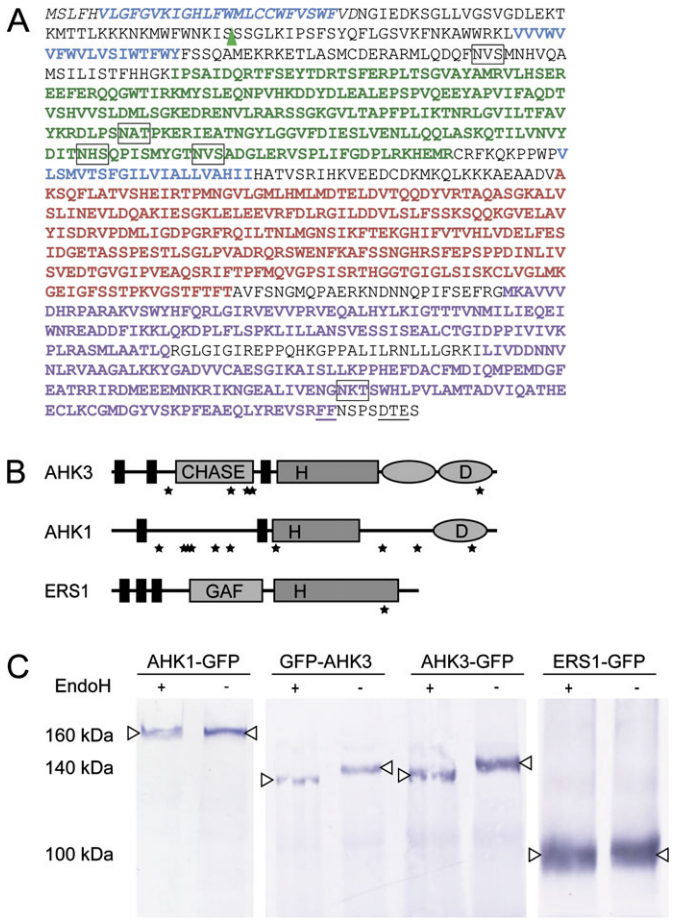

Fig. 6. The GFP fusion proteins of AHK3 show EndoH sensitivity and are glycosylated in vivo. (A) Amino acid sequence of AHK3. The transmembrane domains are shown in blue, the histidine kinase domain in red, and the pseudo receiver domain and the receiver domain in purple. N-X-S/T sequons are framed. The predicted signal peptide is italicized, and the putative ER export signals are underlined. The green triangle marks the site where GFP was inserted into AHK3intGFP. (B) Representations of AHK3, AHK1, and the ethylene receptor ERS1. Putative glycosylation sites are indicated with asterisks. (C) The electrophoretic mobility of AHK3 is endoglycosidase H (EndoH) sensitive. Equal volumes of protein extracts from transiently transformed tobacco leaves expressing the indicated fusion proteins were treated with EndoH (+) or mock treated (–), followed by western blot analysis and immunodetection using an anti-GFP antibody. The fusion proteins are indicated by arrowheads.

the cytoplasm. This topology of the receptor is in agreement with the observation that the binding of the cytokinin zeatin to AHK3 and AHK4 has a pH optimum of ~6.5 (Romanov *et al.*, 2006)—a pH found in the ER lumen (Kim *et al.*, 1998). At pH values of ~5.5—as reported for the apoplast (Li *et al.*, 2005)—the binding of zeatin to AHK3 is almost abolished (Romanov *et al.*, 2006). Thus, when the cytokinin receptors are located in the ER and expose the CHASE domain to the lumen, they bind their ligand with much higher affinity. Furthermore, although the subcellular distribution of active cytokinins and their ability to permeate the cell membrane (Laloue *et al.*, 1981) have not yet been examined in detail, many enzymes involved in cytokinin biosynthesis, such as the isopentenyl transferases (IPTs) and lonely guys (LOGs), and in catabolism, such as cytokinin oxidases (CKXs), are not only found in plastids (IPTs; Kasahara *et al.*, 2004) but also in the cytoplasm and nucleus (LOGs; Kuroha *et al.*, 2009) and other organelles such as the vacuole and the ER (CKXs; Werner *et al.*, 2003). These observations suggest intracellular mechanisms which distribute the hormone and its derivates within the cell. In addition, several plasma membrane-bound carriers have been identified which are able to transport cytokinin into the cell (Burkle *et al.*, 2003; Wormit *et al.*, 2004; Hirose *et al.*, 2005; Cedzich *et al.*, 2008) where it could be distributed further. So apparently the current model of cytokinin signal perception at the plasma membrane needs to be reconsidered.

Recent analyses showed that other hormone perception, signalling, and distribution compounds as well as hormone metabolic enzymes are also found at the ER (Friml and Jones, 2010). For instance, ethylene perception by the five ethylene receptors and their interaction with central downstream signalling elements such as constitutive triple response 1 (CTR1) and ethylene insensitive 2 (EIN2) occur at the ER (Chen *et al.*, 2002; Gao *et al.*, 2003; Grefen *et al.*, 2008; Bisson *et al.*, 2009; Bisson and Groth, 2010). Furthermore, the auxin-binding protein 1 (ABP1) and the PIN-formed 5 (PIN5) auxin efflux carrier localize to the ER (Tian *et al.*, 1995; Chen *et al.*, 2006; Mravec *et al.*, 2009), where they are discussed to be involved not only in auxin homeostasis and metabolism but also in auxin signalling (Friml and Jones, 2010). Hormonal cross-talk decisively contributes to the final physiological and developmental output of hormone action (Benkova and Hejatko, 2009). It is, therefore, intriguing to speculate that the ER might represent the intracellular site for hormonal cross-talk.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. AHK3GFP fusion proteins localize to the ER in transiently transformed *Arabidopsis* cotyledon cells.

Figure S2. GFP fusion proteins of AHK3 do not co-localize with the Golgi marker G-rk CD3-967.

Figure S3. AHK3–GFP fusion proteins do not co-localize with the plasma membrane-localized fusion protein NHL3–RFP.

Figure S4. The ER localization of AHK3–GFP and GFP–AHK3 does not change upon cytokinin treatment.

Figure S5. AHK3–GFP fluorescence is detectable in the ER of the AHK3–GFP-expressing *Arabidopsis* line.

Table S1. Oligonucleotides used in the study.

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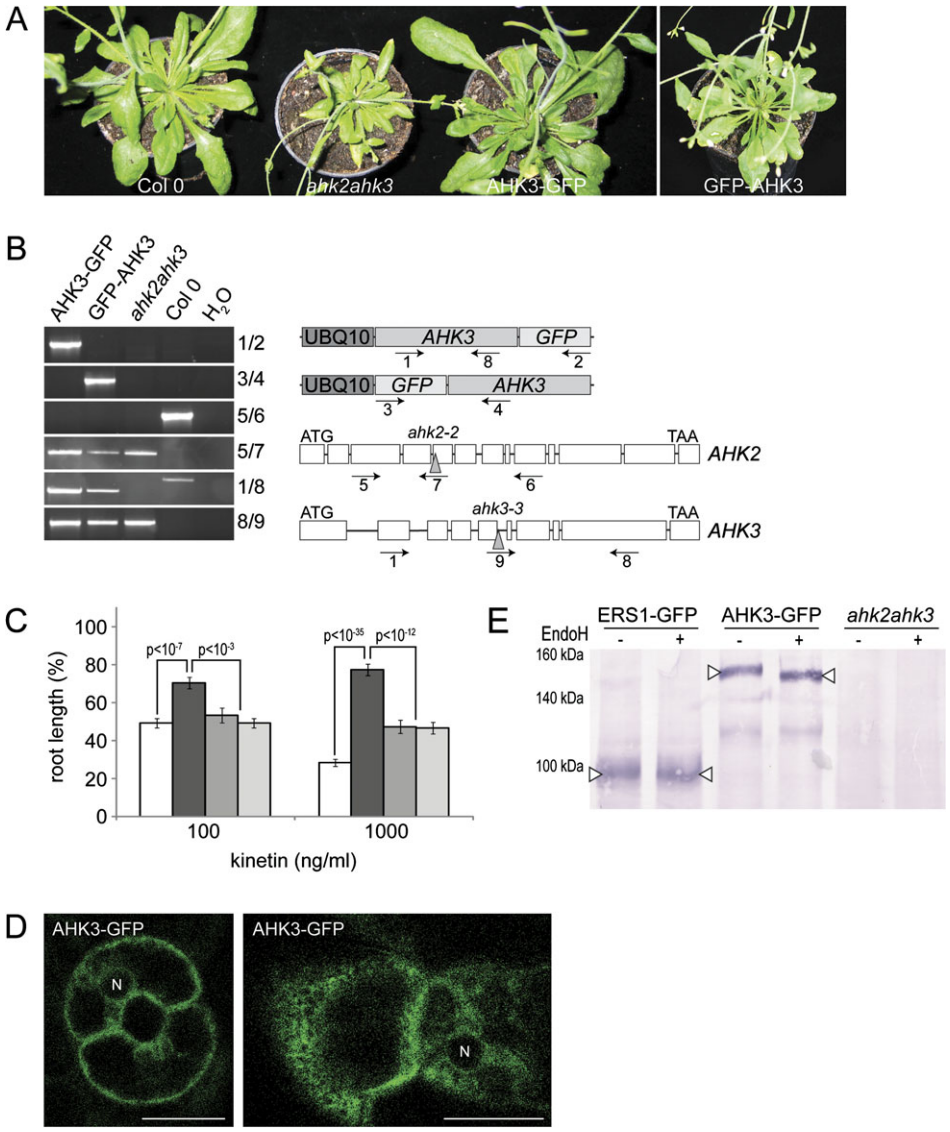

Fig. 7. The GFP fusions of AHK3 are able to complement the mutant phenotype of the *ahk2ahk3* receptor mutant and locate to the ER in the complemented lines. (A) Adult plants of the wild type (Col 0), the *ahk2ahk3* mutant, and plants expressing AHK3–GFP or GFP–AHK3 from the *UBQ10* promoter in the *ahk2ahk3* background (AHK3–GFP and GFP–AHK3). (B) Genotypic analysis of AHK3–GFP and GFP–AHK3 lines by PCR using the indicated primer pairs. (C) Inhibition of root elongation by increasing amounts of exogenously applied cytokinin (kinetin). Seedlings of the wild type (Col 0, white columns), the *ahk2ahk3* mutant (black columns), and the complemented, heterozygous AHK3–GFP and GFP–AHK3 lines (dark and light grey columns) were grown on 0.5× MS agar plates supplemented with the indicated concentrations of kinetin. Mean values (n=18; standard errors) relative to the root length of non-treated controls and P-values for statistical significance are given. (D) AHK3–GFP fluorescence is detectable in the ER of the *AHK3-GFP*-complemented *Arabidopsis* line. CLSM images of epidermis and stomatal cells from cotyledons of etiolated seedlings are shown. N indicates the nucleus. Bars represent 10 μm. (E) EndoH sensitivity of the AHK3–GFP fusion protein in the *AHK3-GFP*-complemented line. Equal amounts of protein extract from seedlings of the indicated lines were treated or not with endoglycosidase H (EndoH), followed by western blot analysis and immunodetection with an anti-GFP antibody. The fusion proteins are indicated by arrowheads.

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