Ligand-binding properties and subcellular localization of maize cytokinin receptors

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Abstract
The ligand-binding properties of the maize (Zea mays L.) cytokinin receptors ZmHK1, ZmHK2, and ZmHK3a have been characterized using cytokinin binding assays with living cells or membrane fractions. According to affinity measurements, ZmHK1 preferred N6-(Δ2-isopentenyl)adenine (iP) and had nearly equal affinities to trans-zeatin (tZ) and cis-zeatin (cZ). ZmHK2 preferred tZ and iP to cZ, while ZmHK3a preferred iP. Only ZmHK2 had a high affinity to dihydrozeatin (DZ). Analysis of subcellular fractions from leaves and roots of maize seedlings revealed specific binding of tZ in the microsome fraction but not in chloroplasts or mitochondria. In competitive binding assays with microsomes, tZ and iP were potent competitors of [3H]tZ while cZ demonstrated significantly lower affinity; adenine was almost ineffective. The binding specificities of microsomes from leaf and root cells for cytokinins were consistent with the expression pattern of the ZmHKs and our results on individual receptor properties. Aqueous two-phase partitioning and sucrose density-gradient centrifugation followed by immunological detection with monoclonal antibody showed that ZmHK1 was associated with the endoplasmic reticulum (ER). This was corroborated by observations of the subcellular localization of ZmHK1 fusions with green fluorescent protein in maize protoplasts. All these data strongly suggest that at least a part of cytokinin perception occurs in the ER.

Key words: Cytokinin, endomembranes, endoplasmic reticulum, maize, microsomes, receptor, sensor histidine kinase, subcellular localization, Zea mays, zeatin.

Introduction
Cytokinins are a class of plant hormones involved in many aspects of plant growth, development, and environmental responses (Mok and Mok, 2001; Sakakibara, 2006; Argueso et al., 2009; Werner and Schmülling, 2009). In the past decade, tremendous progress has been achieved in the study of cytokinin signalling as receptors and components of intracellular signal transduction were identified and characterized (for reviews, see Kakimoto, 2003; Heyl and Schmülling, 2003; Mizuno, 2005; Hwang and Sakakibara, 2006; Müller and Sheen, 2007; To and Kieber, 2008; Bishopp et al., 2009; Romanov, 2009). All known cytokinin receptors belong to the family of sensor histidine kinases and are multi-domain transmembrane proteins. The functional characteristics of cytokinin receptors were studied mainly in Arabidopsis thaliana. In this species, three cytokinin receptors, AHK2, AHK3, and CRE1/AHK4/WOL (AHK4), were identified which directly bind cytokinins (Yamada et al., 2001; Romanov et al., 2006). A live-cell binding assay (Romanov et al., 2005) demonstrated a ligand preference in the AHKs: AHK3 has a higher affinity to trans-zeatin (tZ) than N6-(Δ2-isopentenyl) adenine (iP), but AHK4 shows similar affinities to both (Romanov et al., 2006). This suggests that the two receptors differ in downstream signalling intensity that depends on structural variation of the cytokinin side chain.

In maize (Zea mays), three genes for cytokinin receptors, ZmHK1, ZmHK2, and ZmHK3a, have been identified (Yonekura-Sakakibara et al., 2004). Sequence analysis showed that ZmHK1, ZmHK2, and ZmHK3a are
homologous to the Arabidopsis receptors AHK4, AHK3, and AHK2, respectively. When expressed in Escherichia coli with the ΔresC and cps::lacZ genetic background (Suzuki et al., 2001), ZmHK1 was most sensitive to iP, and less so to tZ and cis-zeatin (cZ). ZmHK2 was most sensitive to tZ and the ribosome. ZmHK3a did not show specific preferences for either iP, tZ, or cZ. In contrast to the Arabidopsis cytokinin receptors, all ZmHKs responded to cZ (Yonekura-Sakakibara et al., 2004), which generally is believed to be inactive or only weakly active. Although the heterologous expression system strongly suggested functional differentiation in terms of recognition of structural variations of cytokinins, differences in the ‘affinity’ between receptor and the ligands in the assay could not be evaluated using the lacZ reporter system.

Perception of plant hormones occurs on the cell surface (plasma membrane) or in intracellular compartments such as the nucleus and endoplasmic reticulum (ER) (Santner and Estelle, 2009). Since the input domain of cytokinin receptors contains putative transmembrane regions, these receptors are thought to be integrated into membranes. However, while the subcellular location of the downstream modules (His-phosphotransfer proteins and response regulators) is established in A. thaliana and maize (Hwang and Sheen, 2001; Imamura et al., 2001; Asakura et al., 2003), the location of the primary receptors is unclear to date. Early concepts presuming that cytokinin receptors are located in the plasma membrane (Ueguchi et al., 2001; Inoue et al., 2001) were based on a bioinformatic approach. Observation of green fluorescence from a chimeric AHK3-GFP fusion protein driven by the cauliflower mosaic virus 35S promoter strongly supported the idea of a localization in the plasma membrane (Kim et al., 2006), but did not exclude alternative possibilities. Biochemical studies of cytokinin receptors, particularly the dependence of ligand–receptor interaction on pH, led to the suggestion that some cytokinin receptors might function inside the cell (Romanov et al., 2006). The knowledge of receptor subcellular localization is important to understand where and how the contact of receptors with hormones occurs in vivo and by which pathway the signal is transmitted towards intracellular targets. Moreover, localization data can provide hints to receptor trafficking which might play a role in signalling cascades (Geldner and Robatzek, 2008).

The cytokinin binding characteristics of cloned maize receptors, studied by a ‘live-cell binding assay’, are reported here and apparent affinity constants are provided. The in vivo distribution of the high affinity cytokinin binding sites and the cytokinin receptor ZmHK1 in subcellular fractions of maize seedling cells was also studied. All data obtained by two-phase partitioning of cytokinin binding sites, immunodetection of ZmHK1 receptors in fractionated membranes, and expression of ZmHK1-GFP fusions in protoplasts, provide evidence for at least a part of cytokinin perception in the ER.

Materials and methods

Plant material and growth conditions

Maize seeds (Zea mays L., Ross 197 AMV) were germinated in the dark at 28 °C on wet filter paper. Roots were harvested 4 d after germination. For experiments with leaves, maize plants were grown in soil for 2 weeks at 28°C, 16/8 h light/dark in a growth chamber.

For tests on maize protoplasts, the suspension-cultured cell line Z86 (Kawaguchi et al., 1991) was grown in Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 1 μg ml⁻¹ thiamine, 10 μg ml⁻¹ KH₂PO₄, 100 μg ml⁻¹ myo-inositol, and 1 μg ml⁻¹ 2,4-dichlorophenylacetic acid, pH 5.6 at 25 °C with shaking (120 rpm) in the dark.

Bacterial spheroplast isolation

E. coli strain KM100 ΔcscD cps::lacZ (Suzuki et al., 2001) was transformed with pIN-III A3 plasmids harbouring reading frames of the maize cytokinin receptor genes ZmHK1, ZmHK2, or ZmHK3a (Yonekura-Sakakibara et al., 2004). Briefly, the coding regions of ZmHK1, ZmHK2, or ZmHK3a were amplified by PCR with primers 5'-CTGATCAGATGGGGGCAAG-TACC-3' and 5'-CCTCGAGTCAAAACGCGGAATCT-3' for ZmHK1, 5'-CGGATCTCAATGACCGTGAAGC-3' and 5'-CCTCGAGCTACTGAACTTGGTCT-3' for ZmHK2, and 5'-GAAGATCTGATGGGTGTCGCAAG-3' and 5'-CCTCGAGCTACTGAACTTGGTCT-3' for ZmHK3a, and cloned into pCR-BluntII-TOPO (Invitrogen). The B/H/XhoI fragment of ZmHK1, BamHII/XhoI fragment of ZmHK2, and BgHII/XhoI fragment of ZmHK3a were ligated into the BamHII/SalI site of pIN-III A3 vectors. Transformants were grown in liquid Luria-Bertani medium supplemented with 50 μg ml⁻¹ ampicillin. For the induction of cytokinin receptors, E. coli transformed with ZmHK1 and ZmHK2 were grown in the presence of 250 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h at 24 °C with shaking at 150 rpm. ZmHK3a-transformed cells were grown in the presence of 25 μM IPTG without shaking for 24 h, and then with shaking at 100 rpm for 16 h.

Spheroplast isolation was performed as described previously by Romanov et al. (2005). Briefly, 40 ml bacterial suspension was precipitated by centrifugation for 10 min at 4000 g at 4 °C. The pellet was re-suspended in 4 ml 0.1 M TRIS-HCl (pH 7.4) and 18% (w/v) sucrose and centrifuged at 4000 g at 4 °C. The pellet was re-suspended in 2 ml 0.1 M TRIS-HCl (pH 7.4) and 10% (w/v) sucrose, and then 2 ml of 0.2 mg ml⁻¹ lysozyme in 4 mM EDTA (pH 7.4) was added. After mixing, the suspension was incubated on ice for 15 min and then centrifuged at 4000 g at 4 °C. The spheroplast pellet was re-suspended in 35 ml 50 mM MES-KOH (pH 7), 150 mM NaCl, 32 mM KCl, and 27 mM NH₄Cl for use in binding assays.

Binding assays

Highly radioisotope-labelled (592 GBq mmol⁻¹) [2-3H]Iz (lI[H]Z) was obtained from the Isotope Laboratory at the Institute of Experimental Botany (Prague, Czech Republic); the radiochemical purity was >99%. For one probe, 3.8 pmol [3H]tZ was used. An aliquot of 750 μl spheroplast suspension was mixed with 2.5 μl of labelled Iz with or without 500-fold excess of unlabelled Iz for the determination of non-specific and total binding, respectively (Romanov et al., 2005). To study the ligand specificity of binding, various unlabelled cytokinins at different concentrations were added together with [3H]Iz. After incubation in an ice bath for 20 min, the mixture was centrifuged at 16 000 g for 3 min at 4 °C. The supernatant was thoroughly removed using a vacuum pump. Two hundred μl 96% (v/v) ethanol was added to the pellet and extracted for 16 h at room temperature in a tightly closed tube. The radioactivity extracted was measured with a scintillation counter.

Plant membrane fractions were incubated with cytokinins in the re-suspending medium. After incubation, microsomes were centrifuged at 16 000 g for 20 min at 4 °C, Mitochondria, chloroplasts, and nuclei were centrifuged at 16 000 g for 3 min at 4 °C.

Membrane fractionation

The plant material was homogenized in a Waring blender with a 2-fold amount of buffer: 50 mM TRIS (pH 7.6 at 22 °C), 20%
glycerol, 150 mM NaCl with either 2 mM EDTA (for subsequent sucrose gradient separation without Mg<sup>2+</sup> and two-phase partitioning) or 5 mM MgCl<sub>2</sub> (for subsequent sucrose gradient separation in the presence of Mg<sup>2+</sup>). (Chen et al., 2002, with minor modifications). Protease inhibitors [15 μM leupeptin, 1 μM pepstatin A, and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)] were added from stock solutions just before homogenization. The homogenate was filtrated through two layers of Miracloth (Calbiochem). The filtrate was centrifuged twice at 10 000 g for 10 min at 4 °C and the supernatant was centrifuged again at 100 000 g for 30 min at 4 °C. The resulting microsome pellets were re-suspended in 10 ml phase buffer [250 mM sucrose, 5 mM K-phosphate buffer pH 7.8, 2 mM dithiothreitol (DTT) for aqueous polymer two-phase partitioning, or 10 mM TRIS (pH 7.6 at 22 °C), 10% sucrose, 1 mM DTT either with 2 mM EDTA or 5 mM MgCl<sub>2</sub> for sucrose gradient fractionation. Pellets were re-suspended using a glass homogenizer (for further fractionation) or by pipetting (for the binding assay as a microsome fraction).

For aqueous two-phase partitioning (Hodges and Mills, 1988), the sucrose concentration was added to a polymer two-phase solution. The two-phase system (6:2%) consisted of 12.4 g 20% (w/w) dextran 500 000, 6.2 g 40% (w/w) polyethylene glycol 3350, 7.5 ml 1 M sucrose in 20 mM K-phosphate buffer, 1 ml 0.8 M NaCl, 10 ml membrane suspension in phase buffer, and was adjusted with water to 40 g total weight. After mixing, tubes containing two-phase mixtures were centrifuged at a rotor speed of 1500 g for 10 min at 4 °C. The same mixtures but without cell membranes (blank tubes) were centrifuged in parallel and used in the following operations. The upper phases from probes with membrane suspension were transferred to new tubes, and each phase was supplemented with its counterpart from a blank tube. The separation was performed three times using pure upper and lower phase solutions (without membranes) obtained in parallel by the same centrifugation step. Then the purified upper (U3, plasma membrane enriched) and lower (L3, ER enriched) phases were 10-fold diluted with phase buffer and centrifuged at 100 000 g for 1 h at 4 °C. Pellets were re-suspended in binding buffer (50 mM MES-KOH pH 7, 150 mM NaCl, 32 mM KCl, 27 mM NH<sub>4</sub>Cl) and used in binding experiments. The lower phases usually contained 15-25-fold more protein than the upper ones.

Sucrose density gradient fractionation
All procedures were carried out at 4 °C. Approximately 100 g of roots were washed with cold deionized water and sliced into segments of about 2 mm length. The segments were homogenized with Polytron (Kinematica) for 30 s in 2 vols (w/v) of buffer A (10 mM TRIS-MES pH 7.5, 250 mM sucrose, 1 mM EDTA, 2.5 mM DTT, 0.5 mM PMSF). The homogenate was filtrated through eight layers of cheesecloth. The filtrate was centrifuged at 1500 g for 10 min at 4 °C. The resulting microsome pellets were re-suspended in 10 ml buffer A and the microsomal fraction was re-suspended in a small volume of buffer A and used for Western blot analysis. In tests that required the presence of Mg<sup>2+</sup>, 5 mM MgCl<sub>2</sub> was added to the buffers.

**Transient expression of ZmHK1-GFP in maize-cultured cells**
The entire reading frame of ZmHK1-GFP was PCR amplified with primers 5'-GGCTGCGAGATGGGGGAAAGT-3' and 5'-CCGCGCATGAAATCTCCTCACCACGGCAATCTC-3' and cloned into pCR-BluntII-TOPO. The SalI/NcoI fragment was ligated into pCaMV35S-sGFP(S65T)-NOS vectors (Sheen et al., 1995; Niwa et al., 1999) to fuse the carboxyl-terminus to the GFP (denoted ZmHK1-GFP). The linker sequence (Gly-Gly-Gly-Ser) was inserted at the conjugation site between the ZmHKs and GFP. Fusion genes of the transmembrane input region of the ZmHKs (ZmHK1, Met1 to Glu347) and GFP were also constructed and designated ZmHK1TM-GFP. The plasmids were introduced into maize suspension-cultured cells by the polyethylene glycol method (Lynzkon and Hodges, 1994). After overnight incubation at 26 °C in the dark, transient expression was observed by confocal laser-scanning fluorescence microscopy (Fluoview IX5, Olympus). Ara6-GFP (N34-GFP) (Ueda et al., 2001) and [endo-xylolucan transferase]-GFP[ER retention signal] (SP-GFP-HDEL, pNMG3) (Takeuchi et al., 2000) were used to indicate the location of plasma membrane and ER, respectively.

**Mitochondrion isolation**
Mitochondrial fractions were prepared from maize cells by the method of Douce et al. (1972) and da Silva et al. (2001), with slight modifications. Maize roots were cut with razor blades and homogenized in a Waring blender (5 s, 3 times, low speed) in the 2-fold amount of buffer A (5 mM TRIS-HEPES pH 7.4, 7 mM cysteine, 300 mM sucrose, 1 mM EGTA, 1 mM PMSF). The homogenate was filtrated through eight layers of cheesecloth. The filtrate was centrifuged at 3000 g for 3 min at 4 °C. The supernatant was centrifuged at 12 000 g for 10 min at 4 °C. The pellet was washed twice with 40 ml of buffer A and then re-suspended in 1.2 ml of buffer A. The resulting crude mitochondrial fraction was purified on a discontinuous sucrose gradient. Layers in the gradient (2 ml each) were 1.8 M, 1.5 M, 1.2 M, 0.9 M, and 0.6 M sucrose in buffer A, topped with 1.2 ml of crude mitochondrial extract (in 0.3 M sucrose). The gradient was centrifuged in a bucket rotor at 40 000 g for 45 min at 4 °C. The fraction on the border between the 1.2 and 1.5 M sucrose layers was accurately pulled out, diluted with buffer A, and centrifuged at 9000 g for 10 min at 4 °C. The purified mitochondrial pellet was re-suspended in buffer A and used in binding experiments.

**Chloroplast isolation**
The chloroplast fraction was prepared from maize cells by the method of Gualberto et al. (1995) with slight modifications. Twenty grams of leaves were homogenized in a Waring blender (twice for 2 s, low speed) in 100 ml of buffer C (0.66 M sorbitol, 0.1 M HEPES-KOH pH 7.3, 4 mM EDTA). The homogenate was filtrated through two layers of cheesecloth and one layer of Miracloth (Calbiochem). The filtrate was centrifuged at 1500 g for 2 min at 4 °C. The pellet was re-suspended in 30 ml of buffer C and centrifuged again. The pellet was re-suspended in 2 ml of buffer C and layered on to a continuous Percoll gradient which had been obtained by mixing 13 ml 2-fold concentrated buffer C with 13 ml liquid Percoll and centrifuging in an angle rotor at 40 000 g for 30 min at 4 °C. The gradient loaded with the sample was centrifuged at 8000 g for 10 min at 4 °C. The green band corresponding to the chloroplast fraction was pulled out, 5e-fold diluted with buffer C, and centrifuged at 2000 g for 5 min at 4 °C. The pellet was re-suspended in buffer C and used in binding experiments.
Nuclei isolation

The nuclear fraction was prepared from maize cells by the method of Saxena et al. (1985) with slight modifications. Thirty grams of roots were homogenized in a Waring blender (twice for 2 s, low speed) in 300 ml of buffer N (250 mM sucrose, 10 mM MES-KOH pH 5.4, 10 mM NaCl, 10 mM KCl, 0.1 mM spermidine, 0.5 mM spermine, 10 mM β-mercaptoethanol, and 5 mM EDTA). The homogenate was filtrated through three layers of Miracloth (Calbiochem) and two layers of nylon mesh 25 μm. Then, 10% Triton X100 was added drop by drop to the homogenate, stirring continuously, to a final concentration of 0.01%. The mixture was incubated for 10 min at 4 °C and then it was centrifuged at 1000 g for 10 min at 4 °C. The pellet was re-suspended in 10 ml of buffer N and layered on to a Percoll step gradient: 10 ml 75% Percoll, 10 ml 25% Percoll in buffer N. The gradient was centrifuged at 1000 g for 40 min at 4 °C. The fraction at the border between the 25% and 75% layers was pulled out, diluted 3-fold with buffer N, and centrifuged at 1000 g for 10 min at 4 °C. The pellet was re-suspended in buffer N and the centrifugation was repeated. Then the pellet of nuclei was re-suspended in 0.44 M sucrose, 50 mM TRIS-HCl pH 7.8, 10 mM β-mercaptoethanol, and 5 mM MgCl2, and was used in the binding experiments. Nuclei dissolved in 1% SDS showed UV spectra typical for nucleoproteins (Bisswanger, 2004).

Monoclonal antibody against ZmHK1

cDNA encoding the receiver-like and the receiver domain of ZmHK1 (Ala651-Val1200) was amplified by PCR with the primers 5′-TGCGATGCTGCTGGTGCATGCCACACTGAAGCACTC-3′ and 5′-TCTCGAGGAAAGTCCGGTCCATCG-3′, and was cloned into the SphI/SalI site of pQE31 (Qiagen). The His-tag recombinant protein was over-expressed in the E. coli M15 strain (Qiagen) and purified using NTA-superose (Qiagen). A monoclonal antibody against the ZmHK1 polypeptide was prepared by Kohjin-Bio (Saitama, Japan). For the evaluation of the specificity of the monoclonal antibody, the corresponding regions of ZmHK2 (Ala695-Gln1007) and ZmHK3a (Ala874-Glu1201) were prepared as His-tag proteins (Thermo Fisher Scientific).

Immunoblot analysis

Goat anti-mouse and anti-rabbit IgG peroxidase conjugates were purchased from Merck (Calbiochem). Equal aliquots (10 μl) were taken from unfrozen fractions for protein separation by 10% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) as described elsewhere (Laemmli, 1970). Prior to SDS-PAGE, protein samples were mixed with SDS-PAGE loading buffer and incubated at 37 °C for 1 h (Schägger, 2006). Proteins were transferred afterwards to Immuno-Blot PVDF transfer membranes (Bio-Rad). For receptor detection, antibodies were diluted 1:3000. Secondary antibodies were diluted 1:10 000 for receptor detection and 1:30 000 for membrane marker detection. Membranes with transferred proteins were preincubated in phosphate-buffered saline (PBS) with 5% skim milk for 1 h; the incubation with primary antibodies was performed in PBS with 5% skim milk for 2 h, then the membranes were washed three times in PBS with 0.05% Tween 20. Incubation with secondary antibodies was performed for 1 h followed by washing three times in PBS with 0.05% Tween 20. The peroxidase reaction was performed using SuperSignalWest Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and the product was detected by CL-XPosure™ Clear Blue X-Ray Film (Thermo Fisher Scientific).

Membrane markers

Polyclonal (rabbit) antibodies against mung bean tonoplast H+-PPase (Takasu et al., 1997), pumpkin BiP (Hatano et al., 1997) and maize plasma membrane H+-ATPase (Nagao et al., 1987) were used for membrane markers.

Affinity constant determination and statistics

Affinity constants were determined on the basis of ligand competition curves using the pharmacology option of the Sigma-Plot program (Systat Software Inc.). Each biological experiment was repeated once or twice and mean values and standard errors were calculated using the t test statistical program.

Results

Live-cell ligand binding assay for maize cytokinin receptors

Live-cell ligand binding assays were performed with recombinant E. coli expressing maize cytokinin receptors. Spheroplasts of three bacterial clones expressing the receptors ZmHK1, ZmHK2, and ZmHK3a were prepared and incubated with [3H]Z alone or mixed with different unlabelled cytokinins. Specific binding was determined; competition curves for six cytokinins (Fig. 1) allowed affinity constants for the interactions of various cytokinins with specific binding sites to be estimated (Table 1). All apparent dissociation constants Kₐ for cytokinins were within the M range, which is reasonable for a high affinity interaction, and were largely close to Kₐ values of Arabidopsis receptors for cytokinin ligands (Yamada et al., 2001; Romanov et al., 2005, 2006). The results also showed peculiarities in ligand specificity for each receptor. ZmHK1 bound tZ and cZ almost equally well with moderate affinity; its affinity to dihydrozeatin (DZ) was very weak (Fig. 1; Table 1). ZmHK2 demonstrated the highest affinities to almost all the cytokinins tested, including DZ; the only exception was 6-benzyladenine (BA) for which ZmHK2 had a lower affinity than ZmHK1 (Fig. 1; Table 1). The properties of ZmHK3a resembled that of ZmHK1 except that ZmHK3a had a higher affinity to tZ than cZ, and BA was a rather moderate competitor (Fig. 1; Table 1). For both ZmHK1 and ZmHK3a, DZ was the weakest competitor. Adenine had no competitive effect.

Ligand-binding assay with maize membrane fractions

An attempt was made to identify the cellular compartment(s) in which cytokinin receptors localize. Having experimental proof for the high affinity perception of [3H]Z in bacteria, binding assays were carried out with subcellular fractions from different organs of maize seedlings. Several subcellular fractions were prepared from maize roots or leaves and tested for the presence of high affinity tZ binding sites. As shown in Fig. 2, purified mitochondria and chloroplasts showed no difference between total and non-specific binding whereas microsome fractions from leaves and roots demonstrated significant specific binding. Nuclei isolated from roots also showed specific binding, although approximately 10-fold less than root microsomes.

Membrane fractionation was achieved by aqueous two-phase partitioning. Immunoblot analysis showed that BiP, an ER marker protein, was abundant in the lower phase while H+-ATPase, a plasma membrane (PM) marker protein, accumulated in the upper phase (Fig. 3A). The distribution of ER and
plasma membrane markers between the two phases was essentially the same for root and leaf membrane fractions. However, binding assays revealed differences in the distribution of affinity sites for tZ in roots and leaves (Fig. 3B). The binding capacity of $[^{3}H]$tZ (per mg protein) was not significantly different between plasma membrane-enriched (U3) and ER-enriched (L3) fractions from roots. Taking into account that the amount of endomembranes in the cell exceeds the amount of plasma membrane by a factor of 15–25 (Morre´ et al., 1987), it could be assumed that most of the high affinity cytokinin binding sites in root cells are located in endomembranes. In leaves, the plasma membrane-enriched fraction (U3) bound approximately 3.5 times more $[^{3}H]$tZ than the ER-enriched fraction (L3) (Fig. 3B), suggesting that the density of tZ binding sites in the leaf plasma membrane-enriched fraction was substantially higher than that in the ER-enriched fraction. Considering a biased protein distribution between the two membrane fractions, it was estimated that no less than 90% and 80% of the total amount of $[^{3}H]$tZ specific binding was attributed to endomembrane fractions from roots and leaves, respectively.

**Table 1.** Apparent affinity constants $K_{D}^*$ for the interaction of different cytokinins with maize receptors $K_{D}^*$ were calculated according to the equation of Cheng and Prusoff (1973; reproduced in Lomin and Romanov, 2008) and expressed in nM. For abbreviations, see legend to Fig. 1.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_{D}^*$ for cytokinins (nM)</th>
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<tr>
<td></td>
<td>tZ</td>
</tr>
<tr>
<td>ZmHK1</td>
<td>24</td>
</tr>
<tr>
<td>ZmHK2</td>
<td>0.4</td>
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<tr>
<td>ZmHK3a</td>
<td>7.2</td>
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**Apparent affinity of cytokinins to leaf and root microsomes**

The ligand specificity of the microsome fractions prepared from roots and leaves was characterized using competitive binding assays (Fig. 4). Microsomes from both organs showed similar properties, i.e. tZ and iP were strong competitors, cZ was less competitive, and adenine showed almost no competition activity. On the basis of the competition curves (Fig. 4), the $K_{D}^*$ values of the microsomes were determined (Table 2). The
KD* values of root microsomes indicated higher affinities to iP and tZ than to cZ. Similar trends were found in leaf microsomes, but all KD* values from leaves were lower than those from roots.

Subcellular localization of ZmHK1-GFP in maize protoplasts

To obtain insights into the subcellular localization of ZmHK1, green fluorescent protein (GFP) fused to the carboxyl-terminus of the ZmHK reading frame (ZmHK1-GFP) or to that of the ZmHK transmembrane input domain (ZmHK1TM-GFP) was expressed in maize cells. Protoplasts were prepared from cultured cells, and the plasmid constructs were introduced into the protoplasts for transient expression under the control of the 35S promoter of the cauliflower mosaic virus (Fig. 5). As controls, GFP alone was used (Fig. 5A) while SP-GFP-HDEL (Takeuchi et al., 2000) and N34-GFP (Ueda et al., 2001) were used as markers of the ER (Fig. 5C) and plasma membrane (Fig. 5E), respectively. The fluorescence signals of ZmHK1-GFP seemed to be co-localized with the ER network and nuclear envelope (Fig. 5G). Essentially the same pattern was observed for ZmHK1TM-GFP (Fig. 5I).

Stable transformants of A. thaliana expressing ZmHK1TM-GFP were also generated. In the T2 progeny, the GFP fluorescence appeared to be co-localized with the ER (Fig. 5K). Taken together, our results suggest that ZmHKs are localized predominantly in the ER membrane.

Immunological detection of ZmHK1 with monoclonal antibodies

To determine more precisely the location of cytokinin perception in membranes, immunological detection of the cytokinin
receptors was performed. First, monoclonal antibodies (mAB) against ZmHK1 were prepared using the receiver-like and receiver domains (Ala651–Val974) as the antigen. Since several genes for cytokinin receptor homologues have been registered recently in genome databases, the reactivity of the mAB was checked. The mAB reacted with ZmHK1, but not with ZmHK2 and ZmHK3a (Fig. 6A). In immunoblot analysis of the root microsome fraction, a single band of around 110 kDa molecular size was detected (Fig. 6B), that is close to the calculated molecular mass of ZmHK1 (108.1 kDa). In aqueous two-phase partitioning of root membranes, H+-ATPase was accumulated in the plasma membrane-enriched fraction (upper phase, U), whereas ZmHK1 was enriched in the microsome fraction (lower phase, L) together with binding protein (BiP) and H+-pyrophosphatase (H+-PPase) (Fig. 6C).

Subcellular fractionation by a sucrose-density gradient centrifugation

The microsomal fraction of the maize root membranes was further fractionated by sucrose density-gradient centrifugation, performed with or without Mg2+. Since the association of the ER with ribosomes is Mg2+-dependent, the depletion of Mg2+ results in ribosome release and a shift of the ER to a lower density in the gradient (Briskin et al., 1987; Chen et al., 2002).

In the presence of Mg2+, which is the condition in which ER-ribosome complexes are stable, the ZmHK1 polypeptide was detected in higher density fractions along with BiP (Fig. 6D). The distribution pattern of H+-ATPase was similar to that of ZmHK1 and BiP, but was shifted slightly to lower densities (Fig. 6D). H+-PPase was distributed in further lower density fractions. The distribution of ZmHK1 was shifted towards lower density fractions when the sucrose density-gradient centrifugation was run in the absence of Mg2+, under which condition ER-ribosome complexes dissociate (Fig. 6D). BiP mostly followed this shift. The distribution of H+-ATPase and H+-PPase remained unchanged. In independent experiments with sucrose-density gradient fractionation, essentially identical results were obtained (see Supplementary Fig. S1 at JXB online). These results support the idea that the ZmHK is predominantly localized in the ER membrane. As for leaves, ZmHK1 was not detected upon membrane fractionation in sucrose gradients (not shown), probably because of its low expression level in leaf cells.

Discussion

Cytokinin receptors from maize were first described by Yonekura-Sakakibara et al. (2004), and the ligand preference of the receptors has been studied by reporter lacZ assays using transgenic E. coli expressing individual ZmHK proteins. In the present study, the same bacterial clones were used to conduct binding assays with highly labelled

Fig. 5. Micrographs of maize protoplasts expressing GFP or various chimeric GF-proteins. GFP alone (A, B), SP-GFP-HDEL (Takeuchi et al., 2000) (C, D), N34-GFP (Ueda et al., 2001) (E, F), HK1-GFP (G, H), and HK1TM-GFP (I, J) were introduced into cultured maize cells by the polyethylene glycol method. After overnight incubation, the cells were examined using a confocal laser-scanning microscope. Stable transformants of A. thaliana expressing HK1TM-GFP (K, L) were generated and the GFP fluorescence in the leaf epidermal cells was observed. (A, C, E, G, I, K) GFP fluorescence; (B, D, F, H, J, L) the corresponding bright field images. Bar=10 μm.
active cytokinin, tZ. ‘Binding preferences’ of receptors do not necessarily have to be consistent with their ‘response preferences’; nevertheless, results of our binding studies (Fig. 1; Table 1) are in good agreement with previously published reporter activation data (Yonekura-Sakakibara et al., 2004). Among the receptors studied, ZmHK1 was distinguished in both assays by its strong preference to iP compared with other cytokinins and by nearly equal responses/affinities to tZ and cZ. ZmHK2 was not very effective in the reporter activation assay. However, the
apparent preference for tZ (Yonekura-Sakakibara et al., 2004) was confirmed in our binding studies (Fig. 1; Table 1). In the case of ZmHK3a, data obtained by both methods were also in close agreement. This overall consistency underlines the importance of the high affinity ligand binding for the subsequent activation of the receptor.

Comparison of cytokinin perception properties from maize and Arabidopsis revealed some common traits. ZmHK2 differed from ZmHK1 by its higher affinity to several cytokinins, namely tZ, cZ, and DZ (Table 1). Analogous differences between AHK3 (orthologue of ZmHK2) and CRE1/AHK4 (orthologue of ZmHK1) have been reported (Spichal et al., 2004; Romanov et al., 2006). An obvious divergence between the two species is the much higher affinity of maize receptors to cZ (Table 1; compare with Romanov et al., 2006). In fact, substantial amounts of cZ and its derivatives (ribosides, glucosides) occur in maize (Veach et al., 2003; Vyroubalova et al., 2009), which implies an important role of cZ in the cytokinin signalling system of maize. Interestingly, cZ-type cytokinins are also abundant in rice (Kojima et al., 2009) and the moss Physcomitrella patens (von Schwartenberg et al., 2007). It is imperative to establish the affinities for cZ of cytokinin receptors in rice and mosses to understand the biological significance of cZ in a variety of taxa. On the other hand, notable differences in the affinity to DZ became apparent in maize cytokinin receptors; the DZ affinity of ZmHK2 was more than two orders of magnitude higher than those of other receptors (Table 1).

Membrane fractionation by aqueous two-phase partitioning revealed a difference in cytokinin binding between membranes from root and leaf cells (Fig. 3). In leaves, tZ binding predominantly occurred in the plasma membrane-enriched fraction, but there was no significant difference in membrane fractions from roots. As the amount of endomembranes in the cell greatly exceeds the amount of plasma membrane (Morrè et al., 1987), this provides evidence for the localization of a majority of the high affinity sites in endomembranes (presumably the ER). Our binding data provided no evidence for high affinity binding sites for tZ in chloroplasts and mitochondria, but suggested some binding in nuclei. At present, there is no unequivocal explanation for the latter finding. Given that the outer envelop of nucleus is contiguous with the ER, co-precipitation of the adjacent endomembranes with nuclei might play a role.

Binding parameters of microsomes (Fig. 4; Table 2) were consistent with the corresponding characteristics of the cloned cytokinin receptors (Table 1). Such agreement is a strong argument for the crucial role of ZmHKs in providing high affinity cytokinin binding sites in cellular membranes. According to the $K_p^*$ values of the cytokinin binding sites in root and leaf membranes, leaf microsomes seemed greatly enriched with ZmHK2 whereas root microsomes evidently harboured a mix of receptors. This conclusion is consistent with existing data on receptor gene expression (Yonekura-Sakakibara et al., 2004; Vyroubalova et al., 2009): the expression of ZmHK2 but not ZmHK1 and ZmHK3a predominated in leaves whereas in roots, ZmHK2 and ZmHK1 were expressed at similar levels while ZmHK3a was also weakly expressed.

Fluorescent imaging of maize protoplasts expressing chimeric GFPs clearly showed the localization of the bulk of the ZmHK1 receptors or its transmembrane domain within the ER network. Similar results were obtained with Arabidopsis stably transformed with the ZmHK1TM-GFP construct (Fig. 5).

Monoclonal antibodies against the cytokinin receptor ZmHK1, in combination with sucrose density fractionation of microsomes, allowed further evidence to be obtained for the localization of this receptor in the ER. Immunoblots (Fig. 6; see Supplementary Fig. S1 at JXB online) confirmed the occurrence of ZmHK1 in cellular membranes and also its predominant location in the ER compared with other membrane types. ZmHK1 co-fractionated with the ER marker BiP and demonstrated a diagnostic Mg$^{2+}$-dependent density shift characteristic for the ER in density gradients. All other membrane types tested (plasma membrane, tonoplast, mitochondrial membranes, Golgi membranes) showed less correlation with the ZmHK1 distribution in sucrose gradients (Fig. 6; see Supplementary Fig. S1 at JXB online). Since plasma membrane H$^+$/ATPase was widely detected in the presence of Mg$^{2+}$, the presence of a small part of the receptors in the plasma membrane is not excluded by our results.

Conclusion

Several lines of evidence presented here suggest that, at least in maize roots, cytokinin receptors predominantly localize to intracellular membranes, particularly to the ER. ER localization has also been suggested for ethylene receptors (Chen et al., 2002), which are structurally related to cytokinin receptors. In addition, the ER localization of all three Arabidopsis cytokinin receptors has been also demonstrated very recently (Wulftange et al. 2011). These suggest that ER plays an important role in plant hormone perception. In the case of cytokinin receptors, the fact of ER localization is not trivial because it was commonly assumed that these receptors are incorporated in the plasma membrane and sense the extracellular hormone (Inoue et al., 2001; Ueguchi et al., 2001). Our data pave the way for understanding the role of intracellular cytokinins. The next important question is whether these internal cytokinin receptors participate in cytokinin signalling or play other role(s). The answer depends on the receptor 3D-structure and orientation in the membranes and the pathway(s) of cytokinin transport to subcellular compartments. It cannot be excluded that the activity of the receptors depends on their subcellular localization. In this context, it should be noted that our two-phase partitioning data indicate organ specificity of cytokinin intracellular localization. Further studies are needed to clarify the above questions.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Fig. S1. Fractionation of maize root membranes by sucrose density-gradient centrifugation.
There are also further data for the Materials and methods (sucrose density gradient centrifugation and membrane markers) and the Results at JXB online.

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