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Antibodies against CKI1$_{RD}$, a receiver domain of the sensor histidine kinase in *Arabidopsis thaliana*: From antigen preparation to *in planta* immunolocalization

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ABSTRACT

Immunodetection is a powerful tool in functional studies of all organisms. In plants, the gene redundancy and presence of gene families composed of highly homologous members often impedes the unambiguous identification of individual gene products. A family of eight sensor histidine kinases (HKs) mediates the transduction of diverse signals into *Arabidopsis thaliana* cells, thereby ensuring the initiation of appropriate adaptive responses. Antibodies recognizing specific members of the HK family would be valuable for studying their functions in *Arabidopsis* and other plant species including important crops. We have focused on developing and applying antibodies against CYTOKININ-INDEPENDENT 1 (CKI1), which encodes a constitutively active membrane-bound sensor HK that regulates the development of female gametophytes and vascular tissue in *Arabidopsis*. A coding sequence delimiting the C-terminal receiver domain of CKI1 (CKI1$_{RD}$) was expressed in *Escherichia coli* using the IPTG-inducible expression system and purified to give a highly pure target protein. The purified CKI1$_{RD}$ protein was then used as an antigen for anti-CKI1$_{RD}$ antibody production. The resulting polyclonal antibodies had a detection limit of 10 ng of target protein at 1:20,000 dilution and were able to specifically distinguish CKI1, both *in vitro* and *in situ*, even in a direct comparison with highly homologous members of the same HK family AHK4, CKI2 and ETR1. Finally, anti-CKI1$_{RD}$ antibodies were able to selectively bind CKI1–GFP fusion protein in a pull-down assay using crude lysate from an *Arabidopsis* cell suspension culture. Our results suggest that the receiver domain is a useful target for the functional characterization of sensor HKs in immunological and biochemical studies.

Introduction

Immunodetection, the detection of proteins using specific antibodies, has become a vital tool in protein functional analysis. The production of specific antibodies recognizing a protein of interest enables the detection and localization of even very small quantities of that protein both on immunoblots (*in vitro*) and in cells and tissues (*in situ*). The high sensitivity of immunodetection is particularly important when studying signaling proteins, whose abundance in any given sample is typically very low. However, the specific identification of proteins that may be functionally divergent despite high structural similarity may still be problematic.

Substantial proportions of proteins in *Arabidopsis thaliana* (and plants in general) are encoded by genes that belong to gene families, including the sensor histidine kinase (HK) family. Sensor HKs are capable of recognizing specific signaling molecules and subsequently initiating downstream signaling cascades via a process known as a multistep phosphorelay (MSP) (Mizuno, 2005). Most plant HKs are hybrid HKs that contain both a histidine kinase

Abbreviations: AA, amino acid; CK, cytokinin; ED, extracellular domain; ER, endoplasmic reticulum; GFP, green fluorescent protein; ELISA, enzyme linked immunosorbent assay; HA, hemagglutinin; HK, histidine kinase; HPt, histidine phospho-transfer protein; IP, immunoprecipitation; MAb, monoclonal antibody; MSP, multistep phosphorelay; PAb, polyclonal antibody; PM, plasma membrane; RD, receiver domain; VB, vascular bundle.

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domain and a receiver domain (RD) in the same protein. Upon signal recognition, a histidine residue in the kinase domain is autophosphorylated and the phosphoryl group is transferred to the C-terminal RD. The RD then communicates with downstream components of the signal transduction pathway via histidine phosphotransfer (HPt) proteins, which receive phosphate groups and relay them to the receiver domains of response regulators in the nucleus (Argueso et al., 2010; Ferreira and Kieber, 2005; Kakimoto, 2003; Lohrmann and Harter, 2002; Mizuno, 2005).

The *Arabidopsis* genome contains genes that encode eight conserved sensor HKs (Schaller et al., 2008). Two of these, ETHYLENE RESPONE 1 (ETR1) and ETHYLENE RESPONSE SENSOR 1 (ERS1), have been identified as ethylene receptors (Hua et al., 1995; Chang et al., 1993). It should be noted that there are three more ethylene receptors in *Arabidopsis*. However, only ETR1 and ERS1 have proven HK activity, while ETR2, ERS2 and ETHYLENE INSENSITIVE 4 (EIN4) seem to be Ser kinases (Moussatche and Klee, 2004). ARABIDOPSIS HISTIDINE KINASE 2 and 3 (AHK2, AHK3), and ARABIDOPSIS HISTIDINE KINASE 4/CYTOKININ RESPONSE 1/WOODEN LEG (AHK4/CRE1/WOL) are cytokinin (CK) receptors (Inoue et al., 2001; Mahonen et al., 2000; Scheres et al., 1995; Suzuki et al., 2001; Yamada et al., 2001). ARABIDOPSIS HISTIDINE KINASE 1 (AHK1) is an osmosensor (Urao et al., 1999) and a positive regulator of drought and salt stress responses (Tran et al., 2007). ARABIDOPSIS HISTIDINE KINASE 5/CYTOKININ-INDEPENDENT 2 (AHK5/CKI2) regulates root elongation, salt sensitivity and pathogen resistance (Iwama et al., 2007; Pham and Desikan, 2012), while the constitutively active HK CYTOKININ-INDEPENDENT 1 (CKI1) has been identified as an essential regulator of female gametophyte development (Hejatko et al., 2003; Pischke et al., 2002) and vascular tissue formation (Hejatko et al., 2009).

CKI1 was originally thought to be involved in CK perception (Kakimoto, 1996). Subsequent studies found no evidence that CKI1 can bind to CKs or is activated in their presence (Yamada et al., 2001), but CKI1 shares some downstream MSP signaling components with the CK signaling pathway (Deng et al., 2010; Hejatko et al., 2009). In addition, the C-terminal RD of CKI1 ($CKI1_{RD}$), which contains a putative phosphorylation site at Asp 1050 (Kakimoto, 1996), is necessary and sufficient for specific protein–protein interactions with downstream members of the MSP signaling pathway in *Arabidopsis* (Pekarova et al., 2011). The crystal structure of $CKI1_{RD}$ retains the $(\alpha/\beta)5$ fold that is characteristic of bacterial and yeast phosphoreceiver domains (Pekarova et al., 2011). This fold is also conserved in the response regulators of simple two-component signaling systems and in both the response regulators and signal RDs of hybrid HKs that participate in MSP signaling pathways (West and Stock, 2001). The backbone structure of $CKI1_{RD}$ is very similar to those of $ETR1_{RD}$ and $AHK5_{RD}$, which are (along with $CKI1_{RD}$) the only plant HK_{RD}s whose structures have been determined (Bauer et al., 2013; Muller-Dieckmann et al., 1999).

Information on the tissue- and cell type-specificity of HK expression and the intracellular localization of these proteins is required for detailed functional characterization of their regulatory roles in *Arabidopsis*. Ethylene receptors, including ETR1, have been found to be localized to the endoplasmic reticulum (ER) (Grefen et al., 2008; Chen et al., 2002). Surprisingly, CK receptors have also been detected predominantly in the ER (Caesar et al., 2011; Wulfetange et al., 2011), but also in the plasma membrane (PM) implying possible involvement in PM/ER shuttling mechanisms. Clearly, fully elucidating the intracellular localization of individual HKs, their tissue specificity and roles would be highly valuable given the probable involvement of MSPs (*inter alia*) in stress tolerance and pathogen resistance in all plants (Argueso et al., 2012; Asakura et al., 2003; Du et al., 2007; Giulini et al., 2004; Choi et al., 2010, 2012; Ito and Kurata, 2006; Jain et al., 2006; Pareek et al., 2006; Tran et al., 2007; Tsai et al., 2012; Yonekura-Sakakibara et al., 2004). Several receptor proteins from the HK family have been identified *in vitro* by immunoblotting protein extracts from overexpressing bacterial, yeast and plant cells, but the probes used in most of these studies were primary antibodies directed against tags or reporters such as GFP, HA or Myc (Caesar et al., 2011; Desikan et al., 2008; Hejatko et al., 2009; Wulfetange et al., 2011). However, when fusion proteins and antibodies directed against a fused tag or epitope are used in localization studies the label may interfere with the natural intracellular distribution of the protein. Thus, it is highly desirable to develop antibodies that directly recognize the protein of interest, as demonstrated for instance in investigations of the localization of auxin transporters of the PINFORMED (PIN) family (Friml et al., 2003; Galweiler et al., 1998; Muller et al., 1998).

To date, antibodies recognizing only a few unlabeled HK sensor peptides or proteins have been developed. The CK receptor AHK4 has been expressed in *Schizosaccharomyces pombe* and detected on immunoblots using αAHK4 antiserum (Yamada et al., 2001). A monoclonal antibody against the ZmHK1 polypeptide, containing both receiver-like and receiver domains, has been used to detect the maize CK receptor ZmHK1 on immunoblots of the maize microsomal root fraction (Lomin et al., 2011). The ethylene receptor ETR1 has been expressed in amounts permitting its immunodetection on blots of proteins from mesophyll protoplasts, *A. thaliana* plants, yeast cells, and *Escherichia coli* (Cho and Yoo, 2007; Qu et al., 2007; Schaller et al., 1995; Voet-van-Vormizeele and Groth, 2003, 2008). However, antibodies raised against untagged proteins have only rarely been used to study the localization of HK sensors within cells or tissues (Hejatko et al., 2009; Chen et al., 2002; Lomin et al., 2011; Xie et al., 2002). The reason for that is a high similarity of sensor HK, which in most cases hampers the production of antibodies specifically recognizing respective HK both *in vitro* and *in situ*.

Here, we report the design, expression, purification and use as an antigen of the $CKI1_{RD}$ protein to develop anti-α$CKI1_{RD}$ polyclonal antibodies. We demonstrate that antibodies raised against $CKI1_{RD}$ can be used to detect CKI1 with high sensitivity and specificity both on immunoblots and *in situ*. Our results imply that receiver domains are suitable targets for producing antibodies specifically targeting potentially every member of the HK_{RD}s family in *Arabidopsis*, which would be valuable tools for elucidation of their distributions, their functions and (hence) HK-mediated signaling pathways in plants.

Results and discussion

Identification of $CKI1_{RD}$

The RDs of hybrid sensor histidine kinases are directly involved in the specific molecular recognition of their downstream signaling partners (Bauer et al., 2013; Inclan et al., 2008; Pekarova et al., 2011; Rogov et al., 2006; Xu et al., 2003; Zhao et al., 2008). Thus, in spite of their conservancy at the AA sequence level, they seem to have distinctive structural features that make them good candidate epitopes for specific antibody production.

To identify the coding region of CKI1 cDNA delimiting $CKI1_{RD}$, we used BLASTP to compare the AA sequence of CKI1 to sequences compiled in several secondary databases (Altschul et al., 1997; Zhang and Madden, 1997). Depending on the database used, $CKI1_{RD}$ was located at the C-terminus of CKI1 at AA positions 986–1114 (SMART (Letunic et al., 2012; Schultz et al., 1998) (Fig. 1A)), 988–1115 (Pfam HMM) (Finn et al., 2010), 987–1085 (Prosite) (Gasteiger et al., 2003) or 989–1117 (RefSeq) (Pruitt et al., 2007).

Cloning, expression and purification of $CKI1_{RD}$ protein

We constructed a plasmid containing the C-terminal fragment of CKI1 (the 179 AA residues located between positions