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# Protein Kinase Domain of CTR1 from *Arabidopsis thaliana* Promotes Ethylene Receptor Cross Talk

**Hubert Mayerhofer, Saravanan Panneerselvam and Jochen Mueller-Dieckmann***

*EMBL Hamburg Outstation, c/o DESY, Notkestr. 85, D22603 Hamburg, Germany*

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Ethylene controls many aspects of plant growth and development. Signaling by the gaseous phytohormone is initiated by disulfide-linked membrane-bound receptors, and the formation of heteromeric receptor clusters contributes to the broad range of ethylene responsiveness. In *Arabidopsis thaliana*, the TCS-like ethylene receptors interact with the cytosolic serine/-threonine kinase constitutive triple response 1 (CTR1), a proposed mitogen-activated protein kinase kinase kinase. In the absence of the hormone, the receptor and therefore CTR1 are active. Hence, ethylene acts as an inverse agonist of its signaling pathway. The three-dimensional structures of the active, triphosphorylated and the unphosphorylated, inactive kinase domain of CTR1 in complex with staurosporine illustrate the conformational rearrangements that form the basis of activity regulation. Additionally, in analytical ultracentrifugation experiments, active kinase domains form back-to-back dimers, while inactive and activation loop variants are monomers. Together with a front-to-front activation interface, the active protein kinase dimers thereby engage in interactions that promote CTR1-mediated cross talk between ethylene receptor clusters. This model provides a structural foundation for the observed high sensitivity of plants to ethylene.



## Introduction

The function of ethylene as a phytohormone was discovered in 1901.[1] Since then, the realization of its profound and multifaceted impact on plant growth and development has been continuously growing.[2] Today, several components of the underlying signaling network are known, but the picture is still incomplete.[3]

In *Arabidopsis thaliana*, the many responses to ethylene are regulated by a group of five membrane-bound receptors (ETR1, ETR2, ERS1, ERS2, and EIN4), which initiate signal transduction.[4] The basic functional unit of the receptors is a disulfide-linked dimer,[5] which binds one copper ion and therefore one ethylene.[6] Non-covalent higher-order complexes between ethylene receptors were also discovered and suggested as an explanation for the broad range of ethylene sensitivity (0.2 nL/L to 1000 μL/L) as well as the dominant nature of ethylene-insensitive mutants.[7] The binding site for ethylene lies within the conserved hydrophobic N-terminal receptor domain,[8] which is located in the membrane of the endoplasmic reticulum.[9] The C-terminal cytosolic domains resemble classical bacterial two-component systems.[10] Two-component systems, which regulate numerous signaling pathways in bacteria, comprise a histidine kinase and a response regulator element (the latter is absent in ERS1 and ERS2).[11] Consistent with this discovery, the autophosphorylation activity of ETR1 from *A. thaliana* was demonstrated *in vitro*,[12,13] but this activity is not essential for *in vivo* signaling.[14]

*Corresponding author. E-mail address: jochenmd@embl-hamburg.de.

Abbreviations used: CTR1, constitutive triple response 1; MAPKKK, mitogen-activated protein kinase kinase kinase; WT, wild type; MS, mass spectrometry; MS/MS, tandem mass spectrometry; AUC, analytical ultracentrifugation; ES, enzyme–substrate; PDB, Protein Data Bank.

Another fundamental member of the ethylene response pathway is constitutive triple response 1 (CTR1), a cytosolic protein kinase, which bears most resemblance to the RAF family of serine/threonine protein kinases.[15,16] RAF family kinases are ordinarily activated by receptor tyrosine kinases and belong to the family of mitogen-activated protein kinase kinase kinases (MAPKKKs), which initiate the cytosolic cascade of many eukaryotic signal transduction pathways.[17] CTR1 is one of only two confirmed direct recipients of ethylene receptor activity. Thus, this signaling pathway presents an interesting case, wherein a two-component signaling system manipulates an MAPKKK and possibly an MAPKKK signaling cascade. EIN2 constitutes another essential, membrane-bound transducer of ethylene signaling.[18] In the absence of ethylene, EIN2 has a short half-life, an effect that appears to depend upon the functional state of the ethylene receptor–CTR1 complex.[19]

Since both ethylene receptors and CTR1 are active in the absence of the hormone, ethylene acts as an inverse agonist of its own pathway. Binding of ethylene inactivates the receptor–CTR1 complex and results in the accumulation of EIN3 and EIL1 (EIN3-like 1) in the nucleus.[20] Consequently, deletion of CTR1 leads to a strong ethylene response in seedlings and adult plants,[17] consistent with its proposed role in the pathway.

CTR1 from *A. thaliana* consists of 821 amino acids ($M_r$ = 90,306) and two domains. The ~540 N-terminal amino acids share little sequence homology with the N-terminus of its closest homologue, B-RAF. This domain interacts with the histidine kinase domains of ETR1 and ERS1.[21] As with other MAPKKKs, deletion of the N-terminal domain of CTR1 leads to a constitutively active kinase.[22,23] The ~280-amino-acid-long C-terminal domain contains all sequence motifs found in serine/threonine kinases and carries 37% sequence identity with human B-RAF.[16] Here, we present the crystallographic structures of the CTR1 kinase domain in its active, threefold phosphorylated and in its inactive, unphosphorylated form. These structures reveal the intramolecular rearrangements that discern the different activity states of CTR1. They also reveal two distinct interfaces that provide a model for CTR1-mediated receptor cross talk.

## Results

### Protein kinase domain of CTR1

The C-terminal and catalytically active protein kinase domain of CTR1 (CTR1-kd) from *A. thaliana* was heterologously expressed in *Escherichia coli* and purified and crystallized as previously described.[24] In addition to the wild-type (WT) construct, we expressed and purified a kinase dead variant of CTR1-kd and characterized both biochemically and crystallographically in order to elucidate the mechanisms, which account for the activation of CTR1-kd. Both constructs start 18 residues before the first glycine residue of the conserved P-loop motif (residue 540 of full-length CTR1). The WT version extends to the natural C-terminus while the inactive kinase lacks the last 10 amino acids, which have been found to be structurally disordered. As expected, CTR1-kd adopts the characteristic protein kinase fold with a five-stranded antiparallel β-sheet in the N-lobe (residues 540–626), including a characteristic α-helix, and a larger, predominantly α-helical C-lobe (residues 632–821). Both lobes are connected by a short linker (residues 627–631) (Fig. 1a).

Crystals of WT CTR1-kd and the kinase dead mutant diffracted X-ray radiation to 3 Å and 2.5 Å resolution, respectively. A summary of the refinement statistics is given in Table 1. Supported by kinetic data, their three-dimensional structures represent the active and inactive conformation of the CTR1 kinase domain.

### Activity of CTR1

CTR1-kd is multiply and heterogeneously phosphorylated when expressed in and purified from *E. coli* (Fig. 2a). Our attempts to produce a homogeneously phosphorylated form of CTR1-kd *in vitro*, by incubating the protein with an excess of ATP, were unsuccessful as shown by mass spectrometry (MS). We performed two different kinds of activity assays to further explore the relationship between structure and activity of CTR1. The first assay is a coupled assay recording the consumption of NADH, which is oxidized as long as the assay contains ADP. The only source of ADP in this assay is CTR1 as long as it consumes ATP. Additionally, the activity was determined with an endpoint assay for ATP using a commercial kit (Promega Kinase-Glo® Plus Luminescent Kinase Assay).[25]

Both assays show the autophosphorylation activity of CTR1-kd, which enters the assays in an already pre-activated state (Fig. 2a). In contrast to the endpoint assay with a defined amount of ATP, the coupled assay regenerates ATP from ADP as part of its pyruvate kinase activity. Our results suggest that the kinase domain of CTR1 is in a dynamic equilibrium where one or several of its side chains undergo rapid phosphorylation and dephosphorylation. Indeed, the consumption of ATP by CTR1-kd in the coupled assay is continuous. Even at initial NADH concentrations, 3 orders of magnitude above the ATP concentration, the nucleotide is turned over until all of the NADH has been consumed. When the assay is resupplied with NADH, its oxidation