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Structural studies of the ethylene signalling pathway in *Arabidopsis thaliana*

Ethylene and its perception

• Endogenous plant hormone that affects many aspects of the plant development as seed germination, fruit ripening or flower senescence as well as responses to disease or wounding

•Signalling occurs via a pathway which resembles the bacterial two-component signalling pathway

•Perception occurs in *Arabidopsis thaliana* via 5 ethylene receptors which act as homodimers but also higher-order non-covalent interaction between different receptors have been reported[1]

• The receptors are located in the Endoplasmic reticulum

• First example of two-component system in higher plants

• Receptors can be subdivided into an N-terminal membrane-spanning ethylene binding domain, a GAF domain of unknown function, a Histidine protein kinase domain and in some cases a C-terminal response regulator domain (Fig. 2)

• 2 receptors, ETR1 and ERS1, posses all the conserved residues considered essential for His kinase activity. The other 3 kinases have a degenerated Histidine kinase motif leading to the separation into subfamily 1 and 2, respectively

• Both subfamilies contribute to the signalling but subfamily 1 seems to play a dominant role

• ETR1 has His Kinase activity, all four other ethylene receptors have serine/threonine kinase activity

Signal transduction

• In the absence of ethylene the receptors interact with the downstream Raf-like kinase CTR1 which represses ethylene responses

• CTR1 has a N-terminal domain of unknown function which interacts with ETR1 and a C-terminal Raf like domain with S/T kinase activity

• Upon ethylene binding the inhibitory effect of CTR1 is alleviated and signalling is proposed to occur via a MAPK pathway to EIN2 and subsequently to the EIN3/EIL transcription factor family (Fig. 1)

• A second signalling mechanism, similar to the two component system found in bacteria, exist which seems to be important for fine tuning

• ETR1 interacts with Histidine containing transfer proteins and influences the transcription of the *Arabidopsis* response regulator 2 which is capable of regulating ethylene response genes[2]

• The role of the histidine Kinase domain and its mode of function are not well understood

Results

• Cloning of domains and combinations thereof of 5 ethylene receptors and the CTR1 S/T kinase domain

• Expression and crystallization trials with 8 successfully expressed domains (partly only with the help of various tags)

• ETR1 dimerization and histidine phosphotransfer domain crystallized in various conditions (all including various PEGs) (Fig. 3)

• Crystals didn't diffract well, optimization screen designed and additive screen used

• Screen didn't significantly improve results, diffraction to 7 Å with non standard pattern (Fig. 3B)

• CTR1 C-terminal S/T Kinase domain expressed using a chaperon containing strain but is phosphorylated at 4 positions in various combinations

• Mutation of the 4 positions to Glu to mimic the phosphorylation, buffer improvement via a Thermofluor screen (10° Tm shift) lead to successful crystallization

• Crystals grew in various conditions but only up to 20µm. Size could be improved using the Opti-salt screen, diffraction only up to 7 Å

• Expression of the complete ETR1 and ERS1 His Kinase domain

• Stable only at low concentrations couldn't be crystallized successfully so far

• Limited proteolysis using Chymotrypsin and Proteinase K give for both domains a stable fragment after 30 min corresponding to the catalytic domain (Fig. 4)

• Remaining constructs are unstable after purification, low yield, fail to concentrate or to give soluble protein or don't produce crystals

Further plans

• Optimization of existing crystals using initial hits and optimization, Opti-salt screens (Qiagen) and changing the constructs

• Surface entropy reduction (optimization of certain exposed residues) of constructs that failed to crystallize so far mutation of 1-2 amino acids on exposed loops[3] to threonine

• Expression of the full length construct including the membrane domain which should be used via small angle X-ray scattering as building scaffold for other domains in case the full length construct can't be crystallized

• Activity assays of the expressed kinase domains

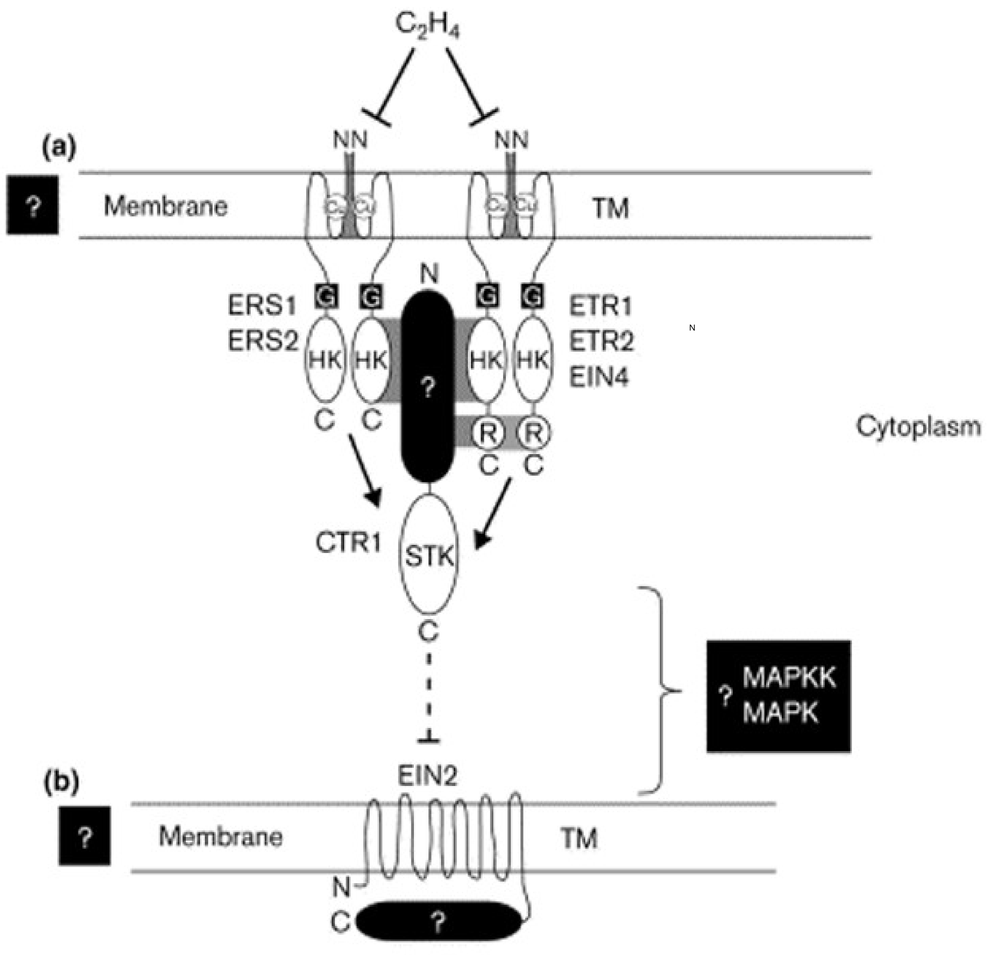

Fig. 1: Ethylene signal transduction pathway with the 5 receptors sitting in the ER membrane interacting with the downstream partner CTR1and furthermore signalling via a putative MAPK pathway

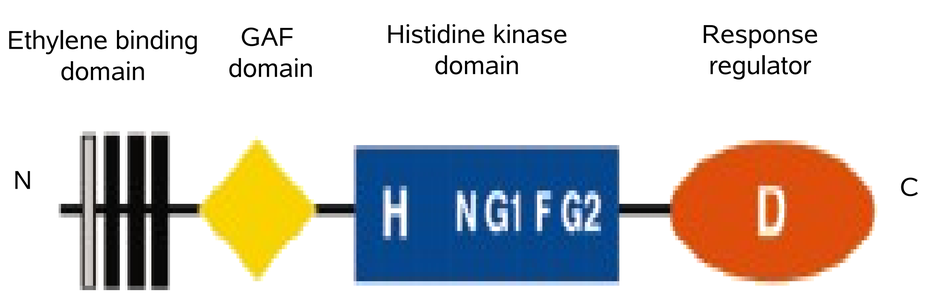

Fig. 2: Domain setup of an ethylene receptors including the N-terminal membrane domain, followed by a GAF and His kinase domain and in some cases by a C-terminal response regulator domain

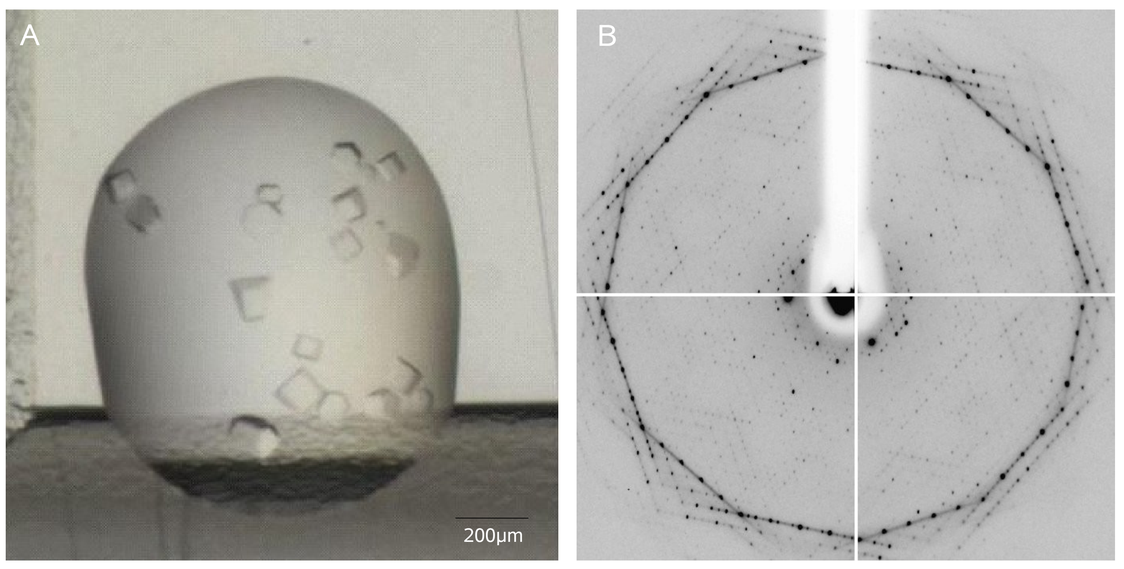

Fig. 3: Crystals obtained from the ETR1 dimerization and histidine phosphotransfer domain (A). The non standard diffraction pattern obtained exhibiting a dodecagon with a ring of strong reflections at 7.5 Å (B)

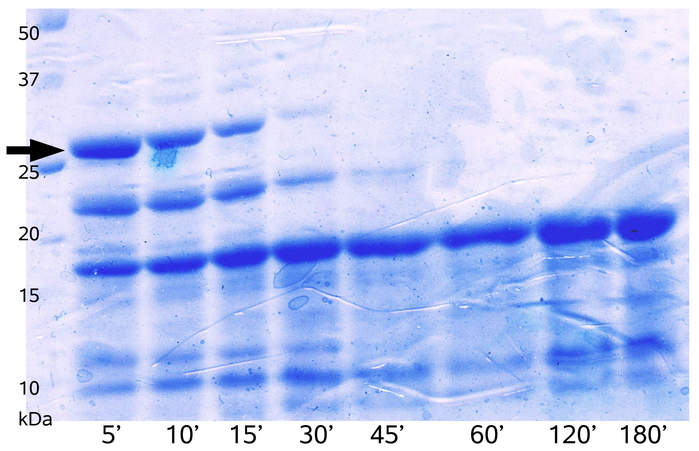

Fig. 4: Limited proteolysis with Chymotrypsin of the ETR1 His Kinase domain which failed to crystallize The arrow indicates the original construct (28kDa). After 30 min a stable fragment (~18kDa) remained

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- Goldschmidt, L., et al., Toward rational protein crystallization: A Web server for the design of crystallizable protein variants. Protein Sci, 2007. 16(8): p. 1569-76