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Perspective

Capturing the Dynamic Movement of Plant Macromolecules by Cryo-Electron Microscopy: Technical Details, First Uses in Photosynthetic Organisms, and Capability to Investigate Crop Physiology

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Abstract: Cryo-electron microscopy (Cryo-EM) is a key technique that allows the visualization of macromolecules in their near native state. Samples are frozen using vitreous ice at −170 °C, eliminating the need for crystallization, as would be required in X-ray crystallography or the use of fixatives in histological procedures. In this perspective paper, we explore the advantages of Cryo-EM over traditional techniques such as X-ray crystallography and nuclear magnetic resonance (NMR), highlighting its ability to capture the dynamics of molecular conformations, up to a limit of 52 KDa, such as viruses, membrane proteins, etc. This paper summarizes how Cryo-EM is changing the view of macromolecular structural biology, with special emphasis on understanding physiological processes common to bacteria, algae, model plants, and crops. It includes a historical perspective, a comparison of advantages (and disadvantages) with other, more classical, techniques, a detailed explanation of the technical details necessary to implement it, both the necessary parameters to be taken into account as well as possible problems, and some pioneer studies in crops as compared to similar studies performed in bacteria, algae, and model plants. In this respect, the most relevant applications for which the technique has been used in order to understand gene regulation, the interaction between membrane receptors and proteins, virus dynamics, and the ability to visualize the formation of self-assembly are described in more detail.

Keywords: crop physiology; cryo-electron microscopy; DNA, RNA and proteins; macromolecules dynamic movement; plant structural biology

1. Introduction

Structural biology advancements have largely contributed to our understanding of plant macromolecules, including proteins, RNA, and DNA. Gaining insights into their structures is crucial for discovering their action mechanisms and functions, with important implications in crop physiology and environmental sustainability. In a world where the population is continuously increasing and climate change threatens global food production and end-product quality, increases in crop productivity are needed to fulfil food demands [1]. One of the keys to success in this challenge will be to perform well-oriented and cutting-edge research [1].

Structural biology progress is fostering our understanding of plant proteins, which are pivotal for essential biological functions. Classic methodologies in protein structural biology, such as X-ray crystallography, are often unable to reveal the dynamics of proteins in their native cellular context. Cryo-EM (single-particle Cryo-EM,

time-resolved Cryo-EM, and cryo-electron tomography) uses transmission electron microscopy (TEM) to visualize macromolecules at extremely low temperature, allowing for to investigation of biological structures in a state close to that existing in nature. In brief, single-particle Cryo-EM is used to determine high-resolution, near-atomic structures of purified biomolecules, time-resolved Cryo-EM is applied to capture and study the dynamic changes and transient functional conformations of biomolecules, whereas cryo-electron tomography determines the biomolecular structures within their native cellular context. Readers are referred to Maurino [2] for more detailed differences among them.

Cryo-EM is an advanced imaging technique used to study the structures of macromolecules, proteins and nucleic acids, at near-atomic resolution (see https://www.ibv.csic.es/servicio-cryo-em/ (accessed on 4 September 2025)). A distinctive feature of this method lies in the sample preparation process: instead of chemical fixation, the samples are rapidly frozen in vitreous ice (a non-crystalline, amorphous form of ice) at extremely low temperatures, often reaching −170 °C. This ultra-fast freezing prevents the formation of ice crystals within the sample, preserving it in a state close to its native conformation. For visualization, TEM is employed to capture high-resolution images of the sample, revealing intricate molecular structures, even down to individual atoms. Moreover, Cryo-EM enables three-dimensional (3D) reconstruction by computationally combining multiple two-dimensional (2D) images captured from different angles. One of the major advantages of Cryo-EM over X-ray crystallography is that it does not require crystallization of the sample, nor the computation of phases necessary for image reconstruction from diffraction patterns (see https://sebbm.es/rincon-del-aula/cristalografia-de-rayos-x-de-macromoleculas/ (accessed on 4 September 2025)).

Cryo-EM is widely applied to visualize biological structures such as viruses, ribosomes, cellular organelles, membrane proteins, and large macromolecular complexes. It provides critical insights into fundamental biological processes that are useful, for instance, in drug development. Importantly, it also allows direct visualization of macromolecular assemblies in their native conformations and within their cellular contexts. This makes it possible to observe dynamic structures such as viral capsids, the bacterial chaperonin GroEL/ES that promotes protein folding post-translation, and larger eukaryotic assemblies like vaults and ferritins (see https://www.ibv.csic.es/servicio-cryo-em/ (accessed on 4 September 2025) and https://sebbm.es/acercate-a/la-crio-microscopia-electronica-para-el-estudio-de-virus-y-aplicaciones-biotecnologicas/ (accessed on 4 September 2025)).

Taking into account this background, this perspective paper aims to describe which techniques were used before Cryo-EM and to understand why Cryo-EM with a dynamic approach started to be used. Furthermore, it will be explained how to carry out the standard protocol as well as the visualization modes and the necessary parameters, including the potential disadvantages of the technique as problems that can occur at the time of analysis. Furthermore, it will be mentioned in which areas the Cryo-EM technique is applicable and the results obtained thanks to this method. Finally, we listed some pioneer studies in crops as compared to similar studies performed in bacteria, algae, and model plants. Among them, the most relevant applications for crops are described in detail.

2. The Cryo-Electron Microscopy Approach from a Historical Perspective

Using Cryo-EM, the first high-resolution structure was reported by Henderson et al. [3]. Twenty-seven years later, Cryo-EM earned Richard Henderson, Jacques Dubochet, and Joachim Frank the 2017 Nobel Prize in Chemistry (https://www.nobelprize.org/prizes/chemistry/2017/summary/ (accessed on 4 September 2025)). Over the past five years, the number of structures resolved by Cryo-EM has increased 5-fold (https://www.rcsb.org/stats/growth/growth-em (accessed on 4 September 2025)), while the number of structures determined by X-ray crystallography has remained fairly stable (https://www.rcsb.org/stats/growth/growth-xray (accessed on 4 September 2025)) [2]. Among all structures determined by Cryo-EM to date, however, only 1.4% correspond to plant proteins (385 plant proteins in total; [2]).

From a historical perspective, Cryo-EM was first used to identify macromolecular complexes acting in gene expression and regulation, and protein biosynthesis. For example, Cryo-EM was used to study spliceosomes. RNA splicing is performed by the spliceosome, a super-protein enzyme complex that removes introns from defined sections of RNA, and abnormal splicing leads to many genetic diseases [4].

Cryo-EM has been key to studying fundamental molecular complexes such as the ribosome and proteasome, involved in protein synthesis and degradation. Thanks to this technique and computational advances, structures of prokaryotic, eukaryotic, and mitochondrial ribosomes have been resolved at high resolution. It has also unravelled structural and functional mechanisms of the proteasome, a cellular machinery essential for the degradation of misfolded proteins and protein regulation [4].

Cryo-EM has also revolutionized the study of membrane proteins such as ion channels, transporters, and supramolecular complexes, which are essential in signaling, transport, and proliferation. Due to the difficulty in

crystallising them, this technique has been crucial for resolving their three-dimensional structures with high precision [4].

Cryo-EM was also applied to the study of proteins associated with neurodegenerative diseases, such as γ-secretase. In 2019, its structure in complex with a fragment of Notch was resolved, providing insight into how this enzyme recognizes key substrates such as the amyloid precursor protein [5].

3. Comparison of Cryo-Electron Microscopy with More Classical Structural Biology Approaches

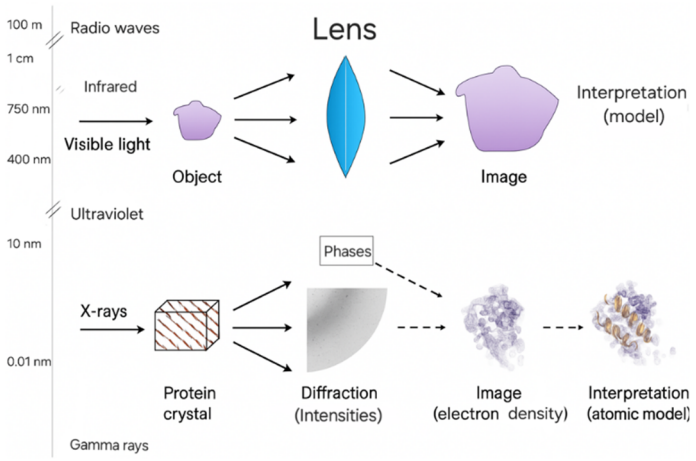
Structural biology studies are mainly carried out by three types of research methods: X-ray crystallography, nuclear magnetic resonance (NMR), and Cryo-EM. Together, they form the basis of high-resolution structural biology research. In May 2025, the Protein Data Bank (PDB) included 235,666 protein structures [2]. Among them, 194,085 (82.4%) were determined by X-ray crystallography, 26,690 (11.3%) by Cryo-EM, 14,522 (6.2%) by nuclear magnetic resonance (NMR) spectroscopy, and 369 (0.1%) using other methodologies [2]. Plant proteins in the PDB remain largely underrepresented; only 2905 (0.01% of the total) are plant protein entries, from which 2379 are X-ray-derived structures, 385 are structures resolved using Cryo-EM, and 141 are derived structures from NMR [2].

X-ray crystallography is applied to proteins with a molecular weight of 10–150 kDa. Thanks to this technique, the vast majority of protein structures have been resolved, which has become the main means of analysing the structure of macromolecules. Although X-ray crystallography dominates research in structural biology, it should be noted that the technique offers static "pictures" of macromolecular structures under equilibrium, and it does not capture transitory states crucial to understanding DNA, RNA, or protein movements occurring during physiological processes. X-ray crystallography struggles to study membrane-associated proteins and large, flexible complexes involved in processes like photosynthesis, respiration, transport, and RNA transduction. Protein flexibility—the ability to change conformation in response to stimuli such as ligand binding, pH, or temperature—is crucial for enzyme function and signal transduction. Examples of highly flexible proteins include enzymes (e.g., triosephosphate isomerase, lactate dehydrogenase), G protein-coupled receptors (GPCRs), and intrinsically disordered proteins. Notably, about 30% of the cellular proteome consists of membrane proteins, including ion channels, transporters, and receptors [6]. An advance of the technique is the concept known as "diffraction before destruction" [7]. It uses ultrashort femtosecond X-ray pulses to record diffraction patterns before the occurrence of radiation damage. This approach, however, has not yet been used for plant proteins [2].

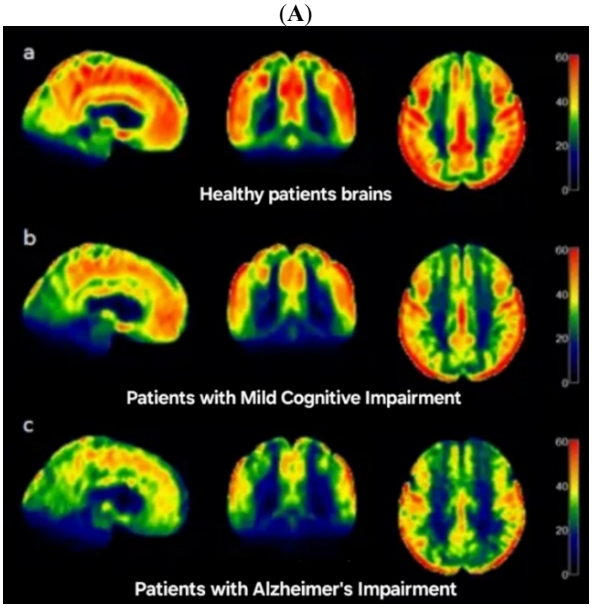
Nuclear magnetic resonance is used to study proteins with a molecular weight of less than 80 kDa. The advantage of this method is that it can determine the structure of protein molecules in solution. However, the application of NMR is limited due to the requirement of high sample concentration (usually at the mM level) and high stability. Nuclear magnetic resonance structures also have a relatively low resolution [4]. Nuclear magnetic resonance spectroscopy determines the 3D protein structure by measuring the magnetic properties of the nuclei in the atoms (^{1}H, ^{15}N, ^{13}C) in a magnetic field, giving information about bond angles, distances between atoms, and chemical environments, which are in turn used to calculate the structure of the target protein [8]. The technique begins with the preparation of isotope-labelled protein samples. The NMR approach is particularly useful for proteins in solution, as mentioned above, enabling research focused on capturing conformational flexibility, allostery, folding, and ligand binding. In plants, the major experimental trouble is the intracellular delivery of isotope-labelled proteins, due to the presence of a rigid cell wall and large vacuoles. However, some attempts have been made with suspension-cultured *Nicotiana tabacum* [9] and *Nicotiana benthamiana* grown hydroponically in a ^{15}N-enriched medium [10].

Recently, the possibility of freezing samples in a thin layer of amorphous ice through advances in cooling methods and rapid vitrification has allowed researchers to develop the so-called Cryo-EM [4]. In the Cryo-EM approach, the samples are rapidly vitrified to preserve their native conformations, then they are imaged by EM, and finally, 2D projections (thousands of them) are computer-processed, reconstructing the protein 3D structure with very high resolution [11]. Thus, the technique enables the observation of macromolecules at near-atomic resolution without requiring crystallization, allowing studies on macromolecules that are difficult or impossible to crystallize, such as membrane proteins or protein complexes large in size [2]. Since thousands of projections are computer-processed at the same time, the technique enables different regions of a macromolecule to move independently of each other or to adopt multiple conformational states, capturing the movements of the macromolecule in its native environment.

Figure 1 shows images that can be obtained from X-ray crystallography (Figure 1A), NMR (Figure 1B), and Cryo-EM (Figure 1C).



(**A**)



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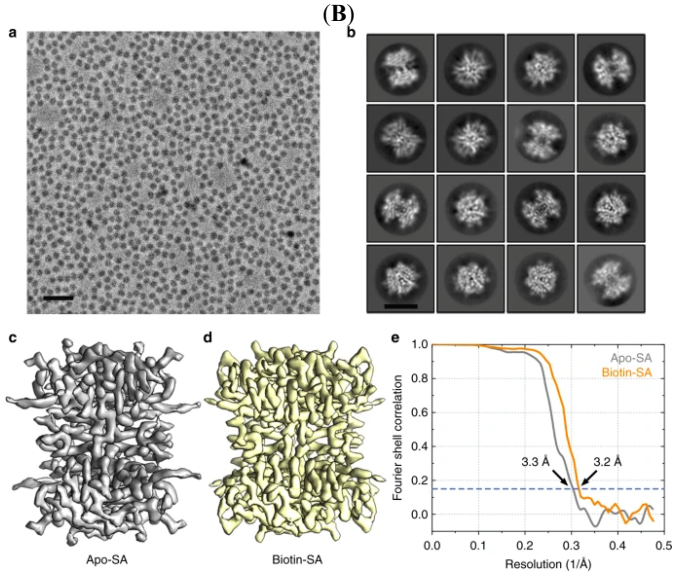

(**C**)

Figure 1. (**A**) Outline, interpretation and image of the X-ray crystallography technique [https://sebbm.es/rincon-del-aula/cristalografia-de-rayos-x-de-macromoleculas/ (accessed on 4 September 2025)]; (**B**) human brain NMR images with progressive cognitive impairment; [https://www.cun.es/nuestros-profesionales/servicios-medicos/radiologia/laboratorio-imagen-biomedica/imagen-alzheimer (accessed on 4 September 2025)]; and (**C**) Cryo-EM reconstruction of individual streptavidin particles (a tetrameric protein that synthesizes the bacterium *Streptomyces avidinii*, characterized by its high affinity for biotin) of 52 kDa at resolution of 3.2 Å (Adapted with permission from [12], Nature Publishing Group, 2019).

4. Benefits of Cryo-Electron Microscopy Compared to NMR and X-Ray Crystallography

Table 1 reports the main advantages and disadvantages of Cryo-EM compared to NMR and X-ray crystallography. For Cryo-EM, the minimum molecular weight limit of the sample particles has been extended to 52 kDa, the resolution has also been improved, and the freezing method has been the most advanced part of the technique. This has made Cryo-EM faster and more efficient, and it can compete with or even replace X-ray crystallography in many respects.

Table 1. Comparative summary of Cryo-EM, X-ray crystallography, and RMN imaging techniques for structural biology studies.

	Cryo-EM	X-ray Crystallography	RMN
Resolution	1.2–4 Å (best on large proteins)	1–2 Å	1.5–3 Å (small proteins)
Sample sensitivity and molecular weight (MW)	- High - MW between 100–500 KDa	- High, but depends on crystallisation - MW between 10–150 KDa	- High for small proteins - Proteins smaller than 80 KDa
Advantages	- No crystallisation required - Study of proteins in the native state - Useful on large proteins (>100 KDa) - Captures multiple conformations	- Atomic resolution - Useful for proteins of all sizes if crystallisable	- Study of proteins in solution - Useful for small proteins (<50 KDa) - No crystallisation required
Disadvantages	- Not ideal for small proteins - Expensive equipment - May require thousands of particles for good resolution	- Requires crystallisation - Does not show natural dynamics, only crystallised conformation - Difficult for membrane proteins	- Size limited → Max 50 KDa - May require isotope labelling - Low structural resolution
Main uses	- Large macromolecular complexes - Membrane proteins - Structures in different conformational states	- Crystallised proteins, either large or small - Protein complexes and ligands	- Small and flexible protein structures - Protein-ligand interactions - Study of molecular dynamics

5. Technical Details: How to Prepare Samples and to Infer Structures in Cryo-Electron Microscopy

For decades, the investigation of macromolecules and related complexes was done by EM. However, quantitative structure determination by 3D reconstruction from EM projections was not feasible unless the molecules were arranged in crystalline order, as in helical symmetry (e.g., bacteriophage tails), arranged in a 2D crystal, or with high symmetry as in viruses, among others [13]. Nevertheless, the continuous development of cryogenics and rapid freezing technology has led to Cryo-EM, or in other words, the microtechnology of using transmission electron microscopy to observe samples at low temperatures. The basic principle of Cryo-EM is to obtain images of biological macromolecules frozen and fixed in vitreous ice, unlike traditional fixation, where misuse or misunderstanding of the fixative could spoil research by losing years of work, thereby obtaining the projection of protein molecules in all directions. Afterwards, by computational use, 3D reconstruction is used to deduce 3D structure from 2D images [4]. Its theoretical basis is the central section theorem proposed by De Rosier and Klug [14]. In simple terms, this theorem states that if you take a 3D object and project it along a certain direction (like shining a light through it), then the Fourier transform of that 2D projection corresponds to a slice through the center of the 3D Fourier transform of the original object, oriented perpendicular to the projection direction (see an example in Figure 1C, subplot e).

Artificial intelligence (AI) and machine learning (ML) are other key actors in Cryo-EM. Perhaps the most prominent application of ML in biology is the prediction of a protein's 3D structure from its amino acid sequence. Transformative tools towards that aim are the development of AlphaFold by Google DeepMind (https://deepmind.google/technologies/alphafold/ (accessed on 4 September 2025)) and RosettaFold (https://github.com/RosettaCommons/RoseTTAFold (accessed on 4 September 2025)). In the former, DeepMind partnered with EMBL's European Bioinformatics Institute (EMBL-EBI) to create AlphaFold DB (https://alphafold.ebi.ac.uk/ (accessed on 4 September 2025)), a freely available resource for the scientific community. In the latter, the RosettaFold code is open and available to the scientific community *via* GitHub. As discussed by Maurino [2], AI and ML also play a crucial role in inferring protein function from sequence and structural information beyond structure prediction. These approaches are used to identify functional regions within proteins, such as active sites, binding pockets, or sites of post-translational modification.

The general process of macromolecular structure analysis by Cryo-EM is, therefore, as follows (Figure 2):

(i) Protein expression and purification: samples with high purity, homogeneity, and integrity must be obtained, and the molecular sieve must show a single peak and symmetrical distribution. Generally, the sample concentration of soluble protein is about 1 mg/mL, and of membrane protein is about 5 mg/mL.
(ii) Negative staining: sample molecules are embedded in a layer of heavy metal salt solution (commonly uranium acetate), so that the heavy metal salt surrounds the molecules.
(iii) Collection of negative staining sample data (sometimes omitting this step depending on the situation).
(iv) Freezing the sample. This is the critical step of the technique and includes two steps: first loading the sample onto the grid to form a thin film of water, and then freezing rapidly. In most cases, the water can be made glassy by immersing the grid in liquid ethane rapidly, by a manual operation reaching temperatures of -170 °C, which has the advantage that the sample can be close to the 'native' state. Because biochemical reactions take place rapidly, another method called rapid mixing/spraying microfluidic chips has had to be developed to obtain the structural information of the intermediate state of the reaction. That is, the two molecular systems are mixed in milliseconds and then rapidly frozen to capture the intermediate steps in biochemical reactions. This method can achieve a time resolution of tens of milliseconds.
(v) Load the sample into the Cryo-EM device.
(vi) Sieve the sample: it is necessary to sieve the sample before data collection to check if the water in the sample is in a glassy state and if the thickness of the ice layer is appropriate. If there is a problem, the sample should be prepared again.
(vii) Data collection: select particles to take electron micrographs, using minimum exposure technology because high-energy electrons cause radiation damage to the sample. Titan Krios, a fully automated cryo-EM from Thermo Fisher Scientific (Waltham, MA, USA), can be used in order to observe the sample.
(viii) Three-dimensional reconstruction.

Some problems may be encountered, however, in the process of using Cryo-EM:

(a) The sample is unstable, degraded, or aggregated.
(b) Some ligands with low molecular weight may not be visible in the density map.
(c) Organic substances such as sugar, DMSO, or glycerine may be present in the buffer, resulting in decreased contrast and resolution of the sample.
(d) The purity of the sample may be good, but the homogeneity poor, which greatly reduces the resolution.
(e) The target region may exhibit higher flexibility, which leads to very poor resolution after 2D or 3D averaging.
(f) Samples can be destroyed during freezing.
(g) In addition to the samples, there are parameters to be optimised, such as sample concentration, blocking time, temperature, grid specifications, etc. [4].

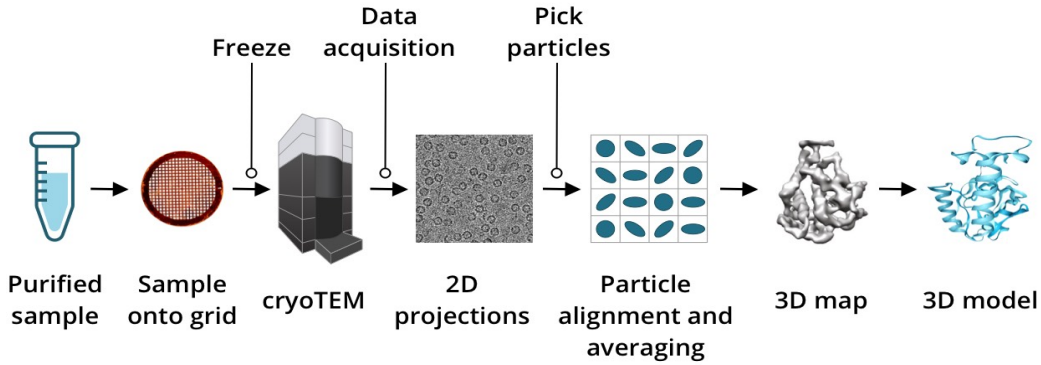

Figure 2. Graphic summary of the Cryo-EM technique procedure (https://staging.myscope.training/CRYO_Introducing_Single_Particle_Analysis (accessed on 4 September 2025)).

6. Some Pioneer Studies of Cryo-Electron Microscopy are Elucidating Crucial Physiological Processes of Photosynthetic Organisms

Although Cryo-EM has been widely developed and applied initially in the field of biomedicine, especially in the study of protein complexes in animals, bacteria, and viruses, in this article, we focus on its growing utility in the study of photosynthetic organisms. Throughout this section, we will prioritise specific examples in which Cryo-EM has been used to resolve relevant structures in plants.

Plants are organisms capable of converting energy through photosynthesis, a process also found in photosynthetic bacteria and algae. Cryo-EM has generated functional insights of membrane proteins participating in the above-mentioned energy conversion, in nutrient uptake and transport, protein biosynthesis, signal transduction, etc. (Table 2).

In bacteria, Cryo-EM was used to resolve the structure of the Rubisco-Rubisco activase (Rca) complex in *Rhodobacter sphaeroides* [15], of phycobilisomes in *Synechocystis* sp. [16], and of light-harvesting II complexes in *Rhodopseudomonas palustris* [17].

In algae, chloroplast native architecture [18], the structure of photosystem I and light harvesting complex I [19], the chloroplast TOC-TIC translocon supercomplex [20] and the major mitochondrial protein complexes [21] from *Chlamydomonas reinhardtii* were resolved.

In model plants, Cryo-EM was able to resolve the structure of photosystem I in state 1 in *Arabidopsis thaliana*, deepening the mechanism of light acclimation during state transitions in higher plants [22], the structure of the respiratory I and III2 supercomplex [23], and the cytokinin transporter AZG1 [24].

In crops, the PSII-LHCII supercomplex of pea [25] and the enzymatically active cytochrome b6f [26] and the thylakoid molecular architecture [27] of spinach were the targets, whereas in *Vigna radiata*, the respiratory I and III2 supercomplex was resolved [28]. In tobacco, the actively translating 80S ribosome structure was elucidated [29].

Let us delve into some pioneer studies in model plants and crops. Using Cryo-EM, Wang et al. [30] were able to resolve the structure of the WeiTsing ion channel (WTS), a protein specifically expressed in the pericycle of *Arabidopsis thaliana* and *Brassica napus* roots during *Plasmodiophora brassicae* infection. The technique allowed them to determine that WTS adopts a pentameric architecture with a central pore, forming a selective channel for cations, particularly calcium (Ca^{2+}) (Figure 3).

This structural information was essential to establishing the relationship between channel form and function. Targeted mutations in key residues, guided by the Cryo-EM model, demonstrated that both oligomerisation and channel activity are indispensable for the activation of immune responses in the plant. Furthermore, it was proposed that WTS acts as a calcium release channel from the endoplasmic reticulum, representing a novel mechanism of immune signalling in plants (Figure 4). Therefore, this study shows how Cryo-EM can be an essential tool for the discovery of new classes of functional proteins and their integration into plant defence pathways.

Table 2. Some examples of experiments carried out with Cryo-EM (single particle and time-resolved Cryo-EM, and cryo-electron tomography) in bacteria, algae, model plants, and crops.

Species	Analytical Technique	Biological/Physiological Process Investigated	Source
Bacteria			
Rhodobacter sphaeroides	Time-resolved Cryo-EM	Rubisco-Rubisco activase (Rca) complex	[15]
Synechocystis sp.	Single-particle Cryo-EM	Phycobilisomes	[16]
Rhodopseudomonas palustris	Single-particle Cryo-EM	Light-harvesting II complexes	[17]
Algae			
Chlamydomonas reinhardtii	Cryo-electron tomography	Chloroplast native architecture	[18]
Chlamydomonas reinhardtii	Single-particle Cryo-EM	Photosystem I and light-harvesting complex I	[19]
Chlamydomonas reinhardtii	Single-particle Cryo-EM	Chloroplast TOC-TIC traslocon supercomplex	[20]
Chlamydomonas reinhardtii	Cryo-electron tomography	Major mitochondrial protein complexes	[21]
Model plants			
Arabidopsis thaliana	Single-particle Cryo-EM	Respiratory I and III2 supercomplex	[23]
Arabidopsis thaliana	Single-particle Cryo-EM	Photosystem I in state 1 (PSI-ST1)	[22]
Arabidopsis thaliana	Single-particle Cryo-EM	Channel for pathogen infection	[30]
Arabidopsis thaliana	Single-particle Cryo-EM	Cytokinin transporter AZG1	[24]
Crops			
Pisum sativum	Single-particle Cryo-EM	PSII-LHCII supercomplexes interactions	[25]
Kalanchoë diagremontiana	Single-particle Cryo-EM	Gene transfer from *Agrobacterium tumefaciens*	[31]
Solanum lycopersicum	Single-particle Cryo-EM	Spotted wilt virus infection	[32]
Vigna radiata	Single-particle Cryo-EM	Respiratory I and III2 supercomplex	[28]
Spinacia oleracea	Single-particle Cryo-EM	Cytochrome b6f is enzymatically active	[26]
Nicotiana tabacum	Single-particle Cryo-EM	Actively translating the 80S ribosome	[29]
Brassica napus	Single-particle Cryo-EM	Channel for pathogen infection	[30]
Glycine max	Single-particle Cryo-EM	Plant-pathogen interaction	[33]
Nicotiana tabacum	Single-particle Cryo-EM	Chloroplast transcription apparatus structure	[34]
Spinacia oleracea	Cryo-electron tomography	Thylakoid molecular architecture	[27]

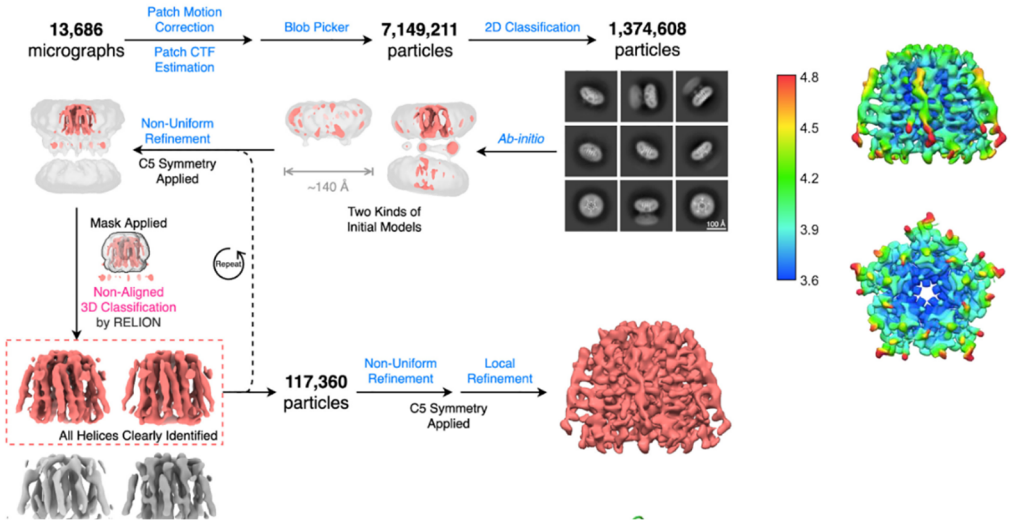

Figure 3. Cryo-EM process used to resolve the structure of the WeiTsing ion channel (Adapted with permission from [30], Elsevier, 2023).

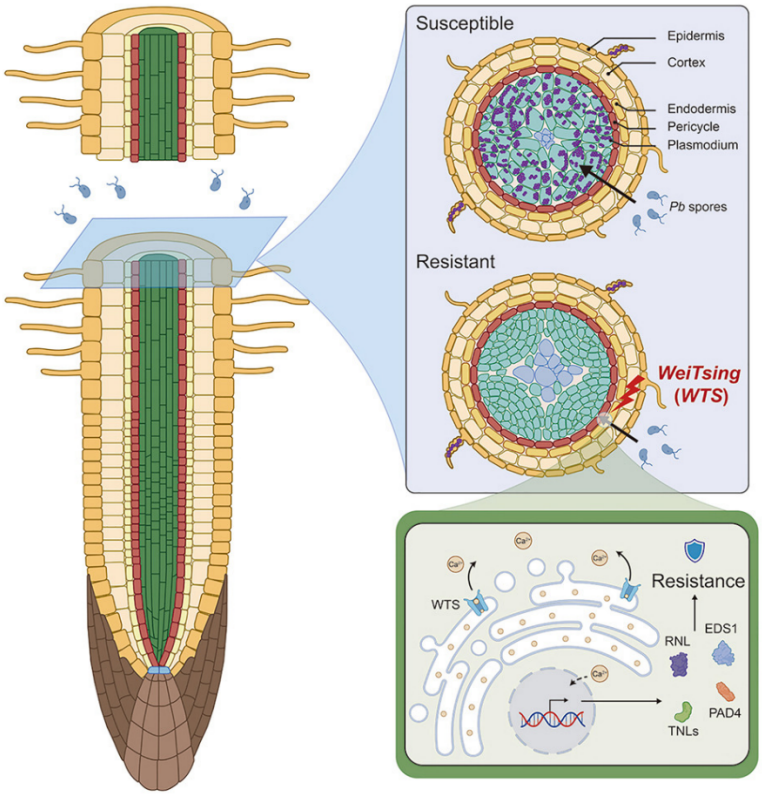

Figure 4. Localization and functioning of the WeiTsing ion channel in plant root (Adapted with permission from [30], Elsevier, 2023).

As an additional example, Cryo-EM allowed Amro et al. [31] to resolve the structure of the *Agrobacterium tumefaciens* T-pilus at a resolution of 3.2 Å, revealing a five-start helical organisation composed of the VirB2 protein and phospholipids. This structure revealed for the first time the presence of phosphatidylcholine within the pilus lumen, which confers a net positive charge to the channel. This property contrasts with other previously studied conjugative pili structures, which were dominated by negatively charged lipids. By targeted mutagenesis, the authors demonstrated that arginine 91 (R91), a positively charged residue exposed to the lumen, is crucial for VirB2 structural stability and pilus formation, as its substitution resulted in loss of assembly and functionality. Thus, the study suggests that the electrostatic charge of the lumen, determined by both amino acids and incorporated lipids, could play a key role in DNA transfer or interaction with the host cell. These findings not only

expand our understanding of the structural diversity of type IV pili, but also underline the value of Cryo-EM as a tool to reveal critical protein-lipid interactions in bacterial nanomachines involved in plant genetic transformation.

Another example is that of Wu et al. [34], who were able to resolve the structure of the PEP-PAP (plastid-encoded polymerase + PEP-associated proteins) plastid transcription complex in *Nicotiana tabacum* at near-atomic resolution. This complex, key in the regulation of gene expression in chloroplasts, is composed of a bacterial catalytic core (PEP) surrounded by fifteen accessory proteins of eukaryotic origin (PAPs). Cryo-EM allowed them to observe how these PAPs are organised into four functional modules: one structural (scaffold), one for antioxidant protection, one for regulation, and one for interaction with RNA. The structure revealed that PAP4 and PAP9 form a module with superoxide dismutase (SOD) activity, responsible for protecting the transcriptional complex from reactive oxygen species. In addition, the PPR domain of PAP2 was shown to recognise specific emerging RNA sequences, suggesting a coupling between transcription and RNA processing. Another relevant finding was that PAP12 occupies a position structurally homologous to the ω-subunit of bacterial RNA polymerase, indicating functional conservation throughout evolution from cyanobacteria. Finally, the resolution of elongating complexes (PEP-PAP-TEC) allowed us to detail the catalytic mechanism of PEP, showing a high similarity to bacterial RNA polymerase, and revealed a structural component unique to plants, the 'b'1 CC gate, which could regulate DNA access in elongation. This work demonstrates how Cryo-EM is essential for understanding the organisation, function, and evolution of key nucleoprotein complexes in plant plastid biology.

Cryo-EM has also been used to investigate plant-pathogen interactions. Plant pathogens represent a significant threat to global agriculture, especially in the context of climate change and reduced agrochemical use. Understanding the molecular interactions that occur between plants and their pathogens is fundamental to developing more effective defense strategies. In this context, Cryo-EM was used to study a key immune recognition complex in different plant-pathogen strains (Figure 5), among which we will focus on the soybean plant.

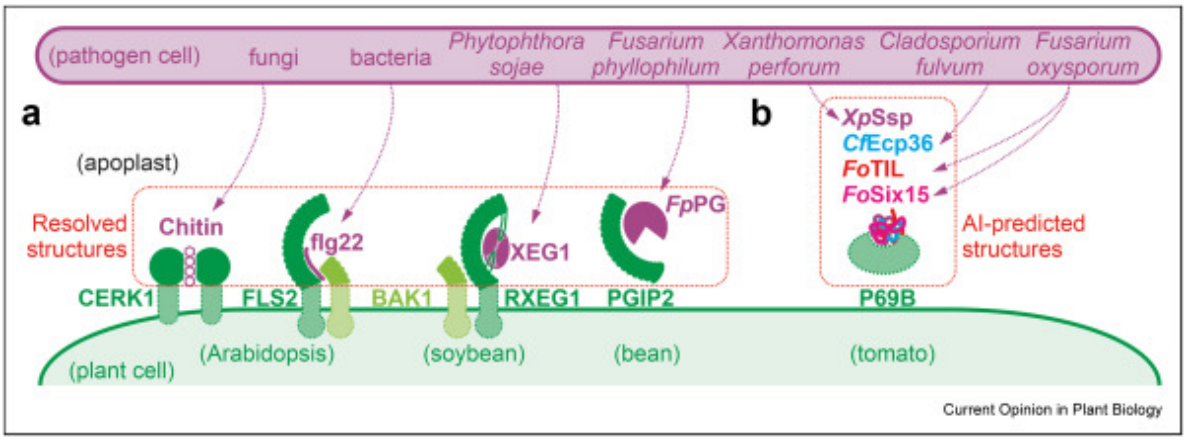

Figure 5. Interactions among plant receptors and pathogen for a variety of plant species and pathogens. (**a**) Resolved structures (purple box) and their context; (**b**) AI-predicted structures of four P69B inhibitors in their context (Adapted with permission from [33], Elsevier, 2024)).

This study focused on the structural characterisation of the complex formed by the *Glycine max* receptor protein RXEG1, its co-receptor BAK1, and the effector XEG1, an endoglucanase secreted by the oomycete *Phytophthora sojae,* facilitating infection by degrading the plant cell wall. Through Cryo-EM, it was possible to visualise how RXEG1 recognises XEG1, thus initiating an immune response in the plant. The analysis revealed that RXEG1 blocks the enzymatic activity of XEG1, suppressing its virulence function. Furthermore, RXEG1 was found to associate with BAK1 via its four C-terminal leucine-rich repeats (LRRs), explaining the role of BAK1 as an essential co-receptor in this defence pathway. Surprisingly, it was observed that XEG1 does not bind directly to BAK1, but induces a conformational change in RXEG1 that enables this interaction.

This work is a good example of sample preparation and data acquisition for Cryo-EM in crops. RXEG1(LRR), XEG1-RXEG1(LRR), and the ternary complex with BAK1(LRR) proteins were purified and concentrated for analysis by Cryo-EM. Samples were applied on perforated carbon grids and vitrified by rapid immersion in liquid ethane. Data acquisition was performed using a Titan Krios TEM operating at 300 kV, with K2 and K3 detectors in super-resolution mode, obtaining thousands of micrographs with a subångström pixel size and an approximate cumulative dose of 50 e-/Å². Image processing and 3D reconstruction (Figure 6) included motion correction (MotionCor2), CTF parameter estimation (CTFFIND4), and particle classification in RELION3.1. More than 4 million particles were selected for RXEG1(LRR) and more than one million for the ternary complex, which were

refined by multiple rounds of classification and 3D reconstruction. High-resolution maps were obtained: 3.21 Å for RXEG1(LRR), 3.11 Å for the XEG1-RXEG1 complex, and 2.92 Å for the complete ternary complex with BAK1. In addition, localised sorting and signal subtraction techniques were applied to improve resolution in specific regions, such as the BAK1 interaction zone, reaching a local resolution of up to 3.54 Å.

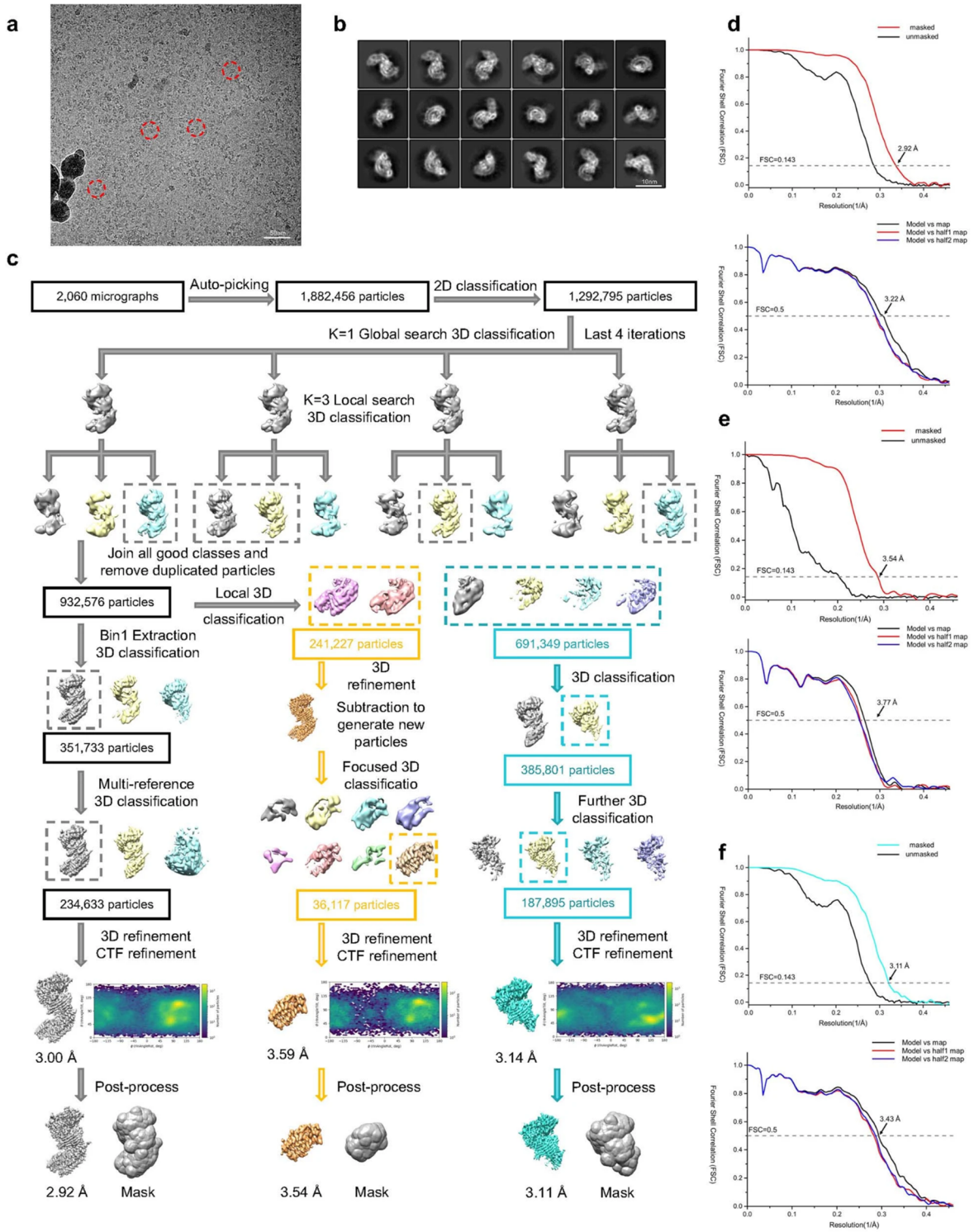

Figure 6. Summary of the process to resolve the XEG1-RXEG1^{LRR}-BAK1^{LRR} ternary complex [35]. (**a**) Representative cryo-EM micrograph of the XEG1-RXEG1^{LRR}-BAK1^{LRR} complex; (**b**) Representative views of 2D class averages of the XEG1-RXEG1^{LRR}-BAK1^{LRR} complex; (**c**) The cryo-EM image processing workflow; (**d**) FSC curves at 0.143 of the final reconstruction of the XEG1-RXEG1^{LRR}-BAK1^{LRR} complex unmasked (red) or masked (black) and FSC curves at 0.5 for model refined against the final map (black), the first half map (blue) and the second half map (red); (**e**) FSC curves at 0.143 and 0.5 of the final reconstruction of BAK1^{LRR}; (**f**) FSC curves at 0.143 and 0.5 of the final reconstruction of the XEG1-RXEG1^{LRR} complex. (Reproduced with permission from [35], Springer Nature, 2025).

The atomic model of the XEG1-RXEG1(LRR)-BAK1(LRR) complex was built by coupling previously available crystal structures (PDB 4MN8) to the Cryo-EM map using Chimera, followed by manual fitting in COOT and refinement in PHENIX. Model validation was performed with tools such as EMRinger, ensuring structural quality. This work provides a detailed molecular basis for how plants perceive pathogen signals and activate their defences through complex receptors. The final result is the characterisation of the RXEG1 receptor interacting with the xyloglucanase XEG1 and the co-receptor BAK1 (Figure 7).

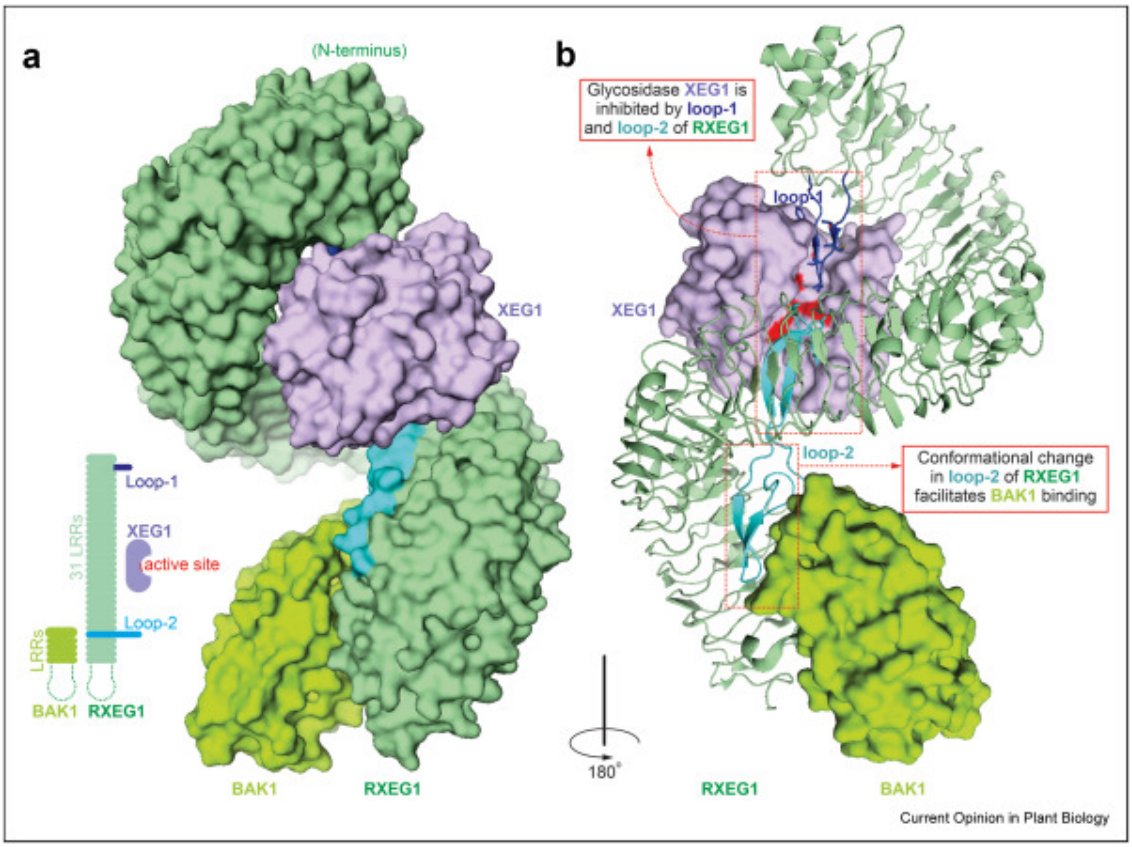

Figure 7. Interaction of the receptor RXEG1 with xyloglucanase XEG1 and coreceptor BAK1. (**a**) Xyloglucanase XEG1 of *Phytophthora sojae* (purple surface representation) interacts with the concave inner surface of the leucine-rich repeats (LRR) of the ectodomain of receptor RXEG1 of *Nicotiana benthamiana* (green surface representation) whilst the ectodomain of coreceptor BAK1 of *Nicotiana benthamiana* (light green surface representation) interacts with the C-terminal LRRs of RXEG1; (**b**) Loop-1 (dark blue) and loop-2 (cyan) of RXEG1 occupy the substrate binding groove of XEG1 (purple surface representation) and inhibit XEG1 activity. XEG1 binding causes a conformational change in loop-2 of RXEG1 that creates a binding site for coreceptor BAK1 (light green surface representation) (Adapted with permission from [33], Elsevier, 2024).

7. Conclusions and Future Applications of Cryo-EM in Structural Biology and Crop Physiology

While Cryo-EM has reached resolutions close to the atomic level, challenges remain to clearly visualise structures smaller than 2 Å. Higher resolution would allow exploration of small complexes, which would have direct implications for pharmaceutical research, enabling more precise design of inhibitors, agonists, or targeted therapies. Integration with AI and structural databases such as PDB, BASEBiO, or cryoSPARC could accelerate the automatic comparison of experimentally derived structures with theoretical models, even facilitating assisted 3D reconstruction.

There are a number of current open questions in plant biology that could be resolved using Cryo-EM, but others that cannot. This technique likely cannot resolve the spatial configuration of 2C protein-phosphatases linked to ABA receptor with or without the presence of ABA, since PP2Cs range 40–45 kDa only, and the ABA receptor itself is 20–30 kDa. 'Hot topics' that, instead, this technique could resolve are, for instance, the configuration of Rubisco in the presence or absence of its night inhibitor, or to distinguish among the spatial configuration of Rubiscos having, e.g., different specificity factors. Similarly, it could resolve the configuration of PSII with or without PsbS, or under different de-epoxidation states of xanthophylls (i.e., under different fluorescence quenching/heat dissipation states).

Cryo-EM will continue to evolve. The number of papers dealing with plant structural biology is expected to largely increase in the coming years, driving science towards in situ and real-time resolution of macromolecular complexes, involving DNA, RNA, and/or proteins, under physiological conditions as they are in the plant cell. From a basic research point of view, Cryo-EM will enable us to observe plant macromolecules in action, observing dynamic processes, such as photosynthesis, respiration, signalling, plant-microbe interactions, and response to abiotic and biotic stress, and other crucial physiological processes. From an applied research point of view, the evolution of the technique will envisage transformative applications in agriculture and crop physiology, environmental-driven plant adaptation and resilience, and sustainable plant biotechnology, guiding the awakening of a new era in a macromolecular structural-informed crop science.

Author Contributions

I.M.-A.: Methodology, investigation and writing original draft; I.M.-A. and F.M.: Writing, review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. Given the role as the Editor-in-Chief, Fermín Morales had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal.

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