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REVIEW

The Big, Mysterious World of Plant 14-3-3 Proteins

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Abstract—14-3-3 is a family of small regulatory proteins found exclusively in eukaryotic organisms. They selectively bind to phosphorylated molecules of partner proteins and regulate their functions. 14-3-3 proteins were first characterized in the mammalian brain approximately 60 years ago and then found in plants, 30 years later. The multifunctionality of 14-3-3 proteins is exemplified by their involvement in coordination of protein kinase cascades in animal brain and regulation of flowering, growth, metabolism, and immunity in plants. Despite extensive studies of this diverse and complex world of plant 14-3-3 proteins, our understanding of functions of these enigmatic molecules is fragmentary and unsystematic. The results of studies are often contradictory and many questions remain unanswered, including biochemical properties of 14-3-3 isoforms, structure of protein–protein complexes, and direct mechanisms by which 14-3-3 proteins influence the functions of their partners in plants. Although many plant genes coding for 14-3-3 proteins have been identified, the isoforms for *in vivo* and *in vitro* studies are often selected at random. This rather limited approach is partly due to an exceptionally large number and variety of 14-3-3 homologs in plants and erroneous *a priori* assumptions on the equivalence of certain isoforms. The accumulated results provide an extensive but rather fragmentary picture, which poses serious challenges for making global generalizations. This review is aimed to demonstrate the diversity and scope of studies of the functions of plant 14-3-3 proteins, as well as to identify areas that require further systematic investigation and close scientific attention.

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INTRODUCTION

14-3-3 proteins are small acidic dimeric proteins found only in eukaryotes. They lack enzymatic activity, but regulate functions of other proteins via binding to specific phosphorylated sites in partner proteins and altering their functional properties.

Mammalian 14-3-3 proteins were first described about 60 years ago; among them, human 14-3-3 proteins have been studied in most detail. The decades of research have led to the understanding that 14-3-3 proteins are involved in the regulation of many fundamental cellular processes, such as intracellular signaling, gene expression, cell cycle control, and cell death. 14-3-3 proteins also play an important role in human and animal diseases, e.g., viral infections and cancer.

Plant 14-3-3 proteins were discovered 30 years later than their mammalian homologs. Because plants are sessile organisms, they must regulate many biochemical processes in order to adapt to constantly changing environmental conditions, so regulatory molecules are of particular importance to them. This is why 14-3-3 regulatory proteins are essential in crucial processes of plant life, such as response to phytohormones, flowering, growth, mineral nutrition, and plant immunity.

Despite the importance of plant 14-3-3 proteins, there are still many unexplored issues, or “blank spots”. Thus, the structure and biochemical properties

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of plant 14-3-3 proteins have been studied much less compared to their mammalian counterparts. An insufficient information on the structure of complexes of plant 14-3-3 proteins with their interaction partners explains the lack of understanding of mechanisms by which 14-3-3 proteins affect the functions of their partners. In most cases, there is no information on the details of 14-3-3 interaction with their partners and effects this binding produces on their activity of the latter. Many 14-3-3 genes have been identified, but the isoforms for *in vivo* and *in vitro* studies are chosen almost at random, and the obtained results provide a very fragmented picture that precludes global generalizations.

This review aims, on one hand, to demonstrate the diversity and broad scope of studies of the functions of plant 14-3-3 proteins, and, on another hand, to highlight the fragmentary nature of current concepts and lack of consistency in the research of plant 14-3-3 proteins.

DIVERSITY AND CLASSIFICATION OF 14-3-3 PROTEINS

Proteins of the 14-3-3 family are common among eukaryotes. They were first isolated in the 1960s from the mammalian brain, where they account for a significant fraction (up to 1% [1]) of all proteins. The name comes from the purification procedure, as these proteins were found in fraction 14 during chromatography on diethylaminoethyl cellulose and produced spot 3.3 during electrophoresis in a starch gel [2]. Further studies have led to the discovery of seven homologous 14-3-3 proteins in mammals that have been designated with the first letters of the Greek alphabet: α, β, γ, δ, ε, ζ, and η. However, the α and δ isoforms were later discovered to be the phosphorylated versions of β and ζ, respectively. We should mention that defining identified 14-3-3 proteins as “isoforms” was incorrect, as each 14-3-3 “isoform” is encoded by a separate gene. Nevertheless, since the term “isoform” has been commonly accepted for describing 14-3-3 proteins, we will use it in our review. Two specifically expressed isoforms, σ and τ (or θ), have been discovered later [3]. 14-3-3 proteins have been rediscovered several times by independent research groups and in different organisms, which resulted in the emergence of alternative names, for example, Leonardo in *Drosophila* fruit fly, BMH1 and BMH2 in *Saccharomyces cerevisiae* yeast, GRF in *Arabidopsis*, etc. [3]. Plant 14-3-3 proteins were discovered almost three decades after their mammalian homologs. In 1992, several papers on plant 14-3-3 proteins were published at the same time [4-7]. Perhaps, no other group of organisms has so many alternative names for 14-3-3 proteins as plants, for example, fusicoccin-binding protein (FCBP) [8], general regulatory factor (GRF) [9], G-box factor 14-3-3 (GF14) [5, 7], nitrate reductase inhibitor protein (NIP) [10], tomato fourteen-three-three (TFT) [11, 12], and rare cold-inducible (RCI) [13]. All these names belong to members of the same protein family. They refer to important biological functions of these proteins and are associated with the history of 14-3-3 protein research. Currently, the names GRF and GF14 are used to classify 14-3-3 proteins from different plant species. Also thirteen 14-3-3 isoforms from the model plant *Arabidopsis thaliana* are named with Greek letters, by analogy with mammalian proteins, but starting from the end of the alphabet: omega (ω), psi (ψ), chi (χ), phi (φ), upsilon (υ), pi (π), omicron (ο), nu (ν), mu (μ), lambda (λ), kappa (κ), iota (ι), and epsilon (ε). The orthologs of *A. thaliana* isoforms from other plants can also be designated with Greek letters, which creates the third system for the naming and classification of plant 14-3-3 isoforms. Table 1 shows the correspondence between the three nomenclatures of 14-3-3 proteins from *A. thaliana*.

Table 1. Nomenclatures for 14-3-3 isoforms from *A. thaliana*

UniProt ID	Greek letters		GRF nomenclature	GF14 nomenclature
P42643	chi	χ	GRF1	GF14χ
Q01525	omega	ω	GRF2	GF14ω
P42644	psi	ψ	GRF3	GF14ψ
P46077	phi	φ	GRF4	GF14φ
P42645	upsilon	υ	GRF5	GF14υ
P48349	lambda	λ	GRF6	GF14λ
Q96300	nu	ν	GRF7	GF14ν
P48348	kappa	κ	GRF8	GF14κ
Q96299	mu	μ	GRF9	GF14μ
P48347	epsilon	ε	GRF10	GF14ε
Q9S9Z8	omicron	ο	GRF11	GF14ο
Q9C5W6	iota	ι	GRF12	GF14ι
F4IA59	pi	π	GRF13	GF14π

Compared to mammalian species, which have seven 14-3-3 isoforms, the number of isoforms encoded by plant genomes can vary significantly. For example, there are 5 isoforms in strawberry (*Fragaria vesca*) [14], 8 in rice (*Oryza sativa*) [15] and cocoa tree (*Theobroma cacao*) [16], 13 in tomato (*Solanum lycopersicum*) [11], 18 in soybean (*Glycine max*) [17],

26 in tea tree (*Camellia sinensis*) [16], and 36 in apple tree (*Malus domestica*) [18].

The existence of numerous orthologs and paralogs, as well as the variability of the total number of 14-3-3 isoforms in different plant species, are due to several whole-genome and segmental duplications that had occurred in plants in the course of evolution [18-22]. It is believed that an ancient duplication has led to the emergence of two separate large 14-3-3 phylogenetic lineages called epsilon group and non-epsilon group. Numerous phylogenetic studies have confirmed this divergence for 14-3-3 isoforms from different plant species [11, 14, 16, 18, 19, 22]. There is no established opinion on the time of the phylogenetic divergence, but it might have occurred before the emergence of green plants [14]. This demarcation of isoforms is fundamental. It can be traced in all studied species of seed plants (Spermatophyta), as each species in this clade has both epsilon and non-epsilon isoforms [14, 19]. The epsilon and non-epsilon isoforms differ in the gene structure. In *A. thaliana*, genes coding for the epsilon isoforms contain 6 to 7 exons separated by 4 to 6 introns, while genes for the non-epsilon isoforms contain 4 exons and 3 to 4 introns [23, 24]. Despite the fact that classification of 14-3-3 paralogs into epsilon and non-epsilon groups is commonly recognized, the authors of some recent studies on the evolution of 14-3-3 proteins do not divide 14-3-3 isoforms into these two large groups [24, 25].

In the course of evolution, large phylogenetic groups have diverged into smaller subgroups. Based on the analysis of gene sequences from three species of monocots and nine species of dicots, Ren et al. [18] identified 11 subgroups (subfamilies) of 14-3-3 genes, four of which were in the epsilon group and seven – in the non-epsilon group [18]. Some subgroups in the study were represented exclusively by 14-3-3 genes from the monocots. In another study, which examined 14-3-3 isoforms from 12 plant species, 4 subgroups were identified in the non-epsilon group, and no subgroups were found in the epsilon group [19]. A very extensive phylogenetic study of the 14-3-3 family analyzed isoforms from 46 species of angiosperms,

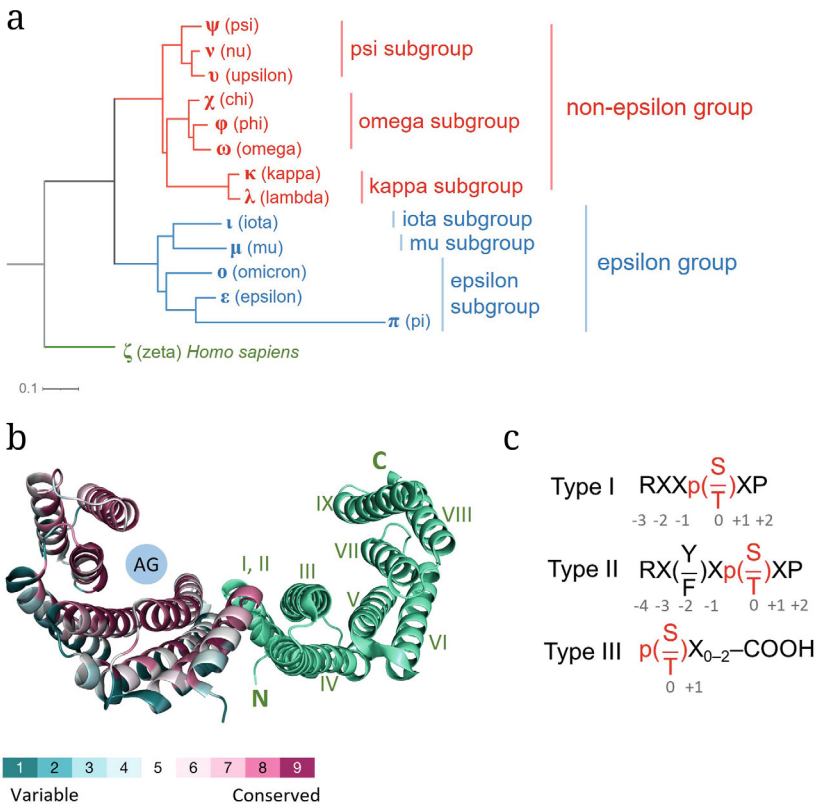

Fig. 1. Phylogeny and structure of plant 14-3-3 proteins. a) Phylogenetic tree of thirteen 14-3-3 isoforms from *A. thaliana*, constructed with MEGA11 using the maximum likelihood method and rooted using human ζ isoform. Evolutionary distance scale (0.1) is shown below. b) Structure of 14-3-3 λ dimer from *A. thaliana* (PDB ID: 8QT5 [26]). The left monomer is colored according to the ConSurf residue conservation score [27] (default settings), with residues conserved across 14-3-3 species shown in magenta and non-conserved residues shown in cyan, with the corresponding color scale below. AG is the amphipathic groove (the binding site for the partner protein phosphorylated sequence). The right monomer is colored green, with the N- and C-termini marked; the alpha-helices are indicated with Roman numerals. c) Amino acid sequences of phosphorylated motifs in 14-3-3 partner proteins. Phosphorylated residues are shown in red. Positions of residues relative to the phosphorylated residue (position 0) are shown in gray.

Table 2. Distribution of plant 14-3-3 isoforms in phylogenetic groups and subgroups (according to Mikhaylova *et al.* [14]).

Systematic groups (according to APG IV)		Family	Plant species	Epsilon group			Non-epsilon group			Epsilon isoforms	Non-epsilon isoforms	Total number of isoforms
				μ	ι	ε	ω	ψ	κ			
Basal angiosperms	Amborellales	Amborellaceae	*Amborella trichopoda*	–	1	1	–	1	1	2	2	10 (6)
Monocots	Zingiberales	Musaceae	*Musa acuminata* (banana)	6	1	1	–	1	1	8	2	10
	Poales	Bromeliaceae	*Ananas comosus* (pineapple)	2	–		3	3	1	2	7	9
			Panicum virgatum (millet)	–	–	3	1	9	-	3	10	13
			Sorghum bicolor (sorghum)	–	–	1	1	4	-	1	5	6
			Hordeum vulgare (barley)	–	–	1	1	5	-	1	6	7
			Triticum aestivum (wheat)	–	–	1	2	10	-	1	12	15 (2)
Dicots	Superasterids	Ranunculales	Ranunculaceae	*Aquilegia coerulea* (aquilegia)	1	2	1	2	1	2	4	5	9
		Caryophyllales	Amaranthaceae	*Amaranthus hypochondriacus* (amaranthus)	3			3	3	2	3	8	11
		Lamiales	Phrymaceae	*Mimulus guttatus* (yellow monkeyflower)	1	2	1	3	2	2	4	7	11
		Solanales	Solanaceae	*Solanum tuberosum* (potato)	2	1	1	4	2	2	4	8	12
				Solanum lycopersicum (tomato)	2	2	1	4	2	2	5	8	13
				Nicotiana tabacum (tobacco)	1	1	2	5	2	2	4	9	13
		Apiales	Apiaceae	*Daucus carota* (carrot)	1	–	–	4	1	3	1	8	9

Table 2 (*cont.*)

Systematic groups (according to APG IV)			Family	Plant species	Epsilon group			Non-epsilon group			Epsilon isoforms	Non-epsilon isoforms	Total number of isoforms
					μ	ι	ε	ω	ψ	κ			
Dicots	Superrosids	Brassicales	Caricaceae	*Carica papaya* (papaya)	2	1	1	1	–	2	4	3	7
			Brassicaceae	*Brassica rapa* (turnip)	1	3	3	7	4	5	7	16	23
				Arabidopsis thaliana	1	1	3	3	3	2	5	8	13
				Arabidopsis lyrata	1	1	1	3	3	2	3	8	11
				Capsella rubella (pink shepherd's purse)	1	1	1	2	3	2	3	7	10
		Sapindales	Rutaceae	*Citrus sinensis* (orange)	2	1	1	2	1	2	4	5	9
		Myrtales	Myrtaceae	*Eucalyptus grandis* (eucalyptus)	1	1	1	1	1	1	3	3	6
		Malvales	Malvaceae	*Theobroma cacao* (cacao)	1	1	1	2	1	1	3	4	7
				Gossypium raimondii (cotton)	1	1	2	8	3	2	4	13	17
		Cucurbitales	Cucurbitaceae	*Cucumis sativus* (cucumber)	1	2	2	2	1	2	5	5	10
		Fabales	Fabaceae	*Glycine max* (soy)	6	3	-	4	3	2	9	9	18
				Trifolium pratense (clover)	4	1	-	1	-	1	5	2	7
		Rosales	Rosaceae	*Prunus persica* (peach)	2	1	1	2	-	2	4	4	8
				Fragaria vesca (strawberry)	1	-	-	1	1	2	1	4	5
		Malpighiales	Salicaceae	*Populus trichocarpa* (poplar)	3	2	2	2	3	2	7	7	14
				Salix purpurea (willow)	2	2	2	2	1	2	6	5	11
			Euphorbiaceae	*Ricinus communis* (castor bean plant)	1	1	1	1	-	1	3	2	5
			Linaceae	*Linum usitatissimum* (flax)	4	3	1	3	2	4	8	9	17

* APG IV (Angiosperm Phylogeny Group) is a current classification system in botany [28].

** Number of non-classified isoforms is shown in parentheses.

including 13 monocots, 32 eudicots, and one representative of basal angiosperms [14]. The epsilon group was divided into three subgroups (iota, mu, and epsilon), and also three subgroups were distinguished within the non-epsilon group (omega, psi, and kappa). This classification was based on the phylogenetic relationships of *A. thaliana* isoforms, and the subgroups were named according to the Greek letter designations of the most characteristic isoforms. The distribution of 14-3-3 isoforms in *A. thaliana* was the following: 5 genes were in the epsilon group [one (ι) in the iota, one (μ) in the mu, and three (ο, π, ε) in the epsilon subgroups] and 8 genes were in the non-epsilon group [three (χ, φ, ω) in the omega, three (υ, ν, ψ) in the psi, and two (κ, λ) in the kappa subgroups] (Fig. 1a). As can be noticed from Fig. 1a, each subgroup contained at least one isoform, making *A. thaliana* a convenient model for a systematic study of 14-3-3 family representatives. The classification based on the 14-3-3 isoforms of *A. thaliana* is complete and the most representative, and we will be using it further in this review.

Numbers of isoforms in subgroups, as well as the representation of subgroups themselves in plants may vary significantly. For example, legumes (Fabaceae) lack the epsilon subgroup, while the iota and epsilon subgroups are absent in carrot (*Daucus carota*) (Table 2). Representatives of the class Monocotyledoneae stand out in terms of the isoform distribution among subgroups. All analyzed representatives of this class, with the exception of banana (*Musa acuminata*), lacked isoforms of the iota subgroup. Plants of the Poaceae family (grasses) also lack the mu and kappa subgroups, but the number of isoforms in the psi subgroup is greatly increased, e.g., up to 10 in wheat (*Triticum aestivum*) (Table 2). In cereals, the non-epsilon group contains only ω isoforms. Such distribution has led to the fact that the psi subgroup has the largest number of isoforms among the analyzed plants, while omega is the most widespread subgroup [14].

The high variability in the number and phylogenetic affiliation of 14-3-3 paralogs among plant species, together with the evolution of these proteins through multiple duplications, have created grounds to assume that the functions of 14-3-3 isoforms are redundant and can overlap. Thus, experiments in yeast *S. cerevisiae* have revealed a high degree of functional similarity between 14-3-3 proteins. *S. cerevisiae* contain only two 14-3-3 genes: BMH1 and BMH2 [29-31]. Knocking out either of them altered the phenotypes of the resulting mutants only slightly compared to the wild-type cells, while the double knockout of both genes was lethal [29, 31]. However, introduction of plant 14-3-3 gene to the double knockout mutants rescued a viable phenotype, which indicated interchangeability of 14-3-3 proteins even from systematically distant groups [32]. However, evolutionary analysis of various angiosperm species revealed the effect of purifying selection on 14-3-3 proteins, which may be associated with an acquisition of a specific function or functions by them [18, 24, 33-35].

Phenotypic analysis of mutant plants deficient by particular 14-3-3 isoforms can shed light on specific functions of these proteins *in vivo* or their redundancy; however, such studies are often unsystematic and incomplete. Even for the model organism as *A. thaliana*, there is no description of phenotypes of mutants deficient by each of all 13 14-3-3 isoforms, which hinders elucidation of their *in vivo* functions. The studies are often focused on a particular feature of mutant plants, while neglecting the other traits. In their extensive analysis of mutations in plant 14-3-3 isoforms, van Kleeff et al. [36] produced single, double, triple, and quadruple knockout mutants for 14-3-3 isoforms from the non-epsilon group (λ, κ, ν, υ, φ, and χ) in *A. thaliana*. The authors found that the length of the main root in single and even double mutants did not differ from that in the wild-type plants; in six triple and three quadruple knockouts, the main root was shorter than in the wild-type plants [36]. When three 14-3-3 isoforms from the epsilon group (ε, μ, and ο) were knocked down simultaneously, the mutant plants demonstrated serious growth impairments and reduced length of the root and the hypocotyl [37]. Only the knockout of the μ isoform (but not the ν isoform) resulted in the reduced root length in *A. thaliana* plants grown at constant illumination; however, both μ- and ν-deficient mutants had shorter roots when grown under red light [38]. The knockout of the μ isoform in *A. thaliana* reduced the length of lateral roots, while the overexpression of this isoform increased it [39]. The transition to flowering under the long-day conditions was delayed in single μ or ν knockout mutants [40]. Overexpression of the GF14c isoform from rice (*O. sativa*) also delayed flowering, while its knockout caused an earlier transition to flowering compared to the wild-type plants [41]. Overexpression of the Me14-3-3VII isoform from cassava (*Manihot esculenta*) in *A. thaliana* increased the content of starch and sugar in the leaves [42]. However, overexpression of another cassava isoform, Me14-3-3II, decreased the amount of starch in the mutant plants [43]. The antisense-mediated knockdown of 14-3-3 ε and μ resulted in a 2 to 4-fold increase in the amount of accumulated starch in the mutant plants compared to the wild-type controls [44]. Overexpression of the SiGRF1 isoform from the fox millet (*Setaria italica*) in *A. thaliana* caused an earlier transition to flowering under the salt stress conditions [25]. Overexpression of the MdGRF13 isoform from apple tree (*M. domestica*) in *A. thaliana* increased plant tolerance to drought and salt stress [18]. Overexpression of another apple

isoform (MdGRF6), on the contrary, led to the increase in the plant sensitivity to exogenous salt, while the knockdown of this isoform induced plant tolerance to salt stress [45].

Apparently, mutations in 14-3-3 proteins mostly affect plant growth (especially root growth), timing of transition to flowering, tolerance to salt stress, and starch accumulation. The phenotype of 14-3-3 mutant plants is determined by the influence of these proteins on specific physiological and biochemical reactions, which will be discussed below.

BIOCHEMICAL CHARACTERISTICS OF PLANT 14-3-3 PROTEINS

Structure of 14-3-3 proteins. Members of the 14-3-3 family are acidic proteins (pI ~ 4-5) with a molecular weight of ~30 kDa. In cells, they typically exist as homo- or heterodimers consisting of the same or different subunits, respectively. The monomers bind to each other in an antiparallel fashion through their N-terminal regions, with the formation of a W-like structure that has the central symmetry and two phosphopeptide-binding (amphipathic) grooves, one in each monomer (Fig. 1b). Both plant and animal 14-3-3 proteins consist of 9 α-helices, each located antiparallel to a previous one. Helices 1-4 of the monomers (N-terminal regions) form a surface necessary for dimerization, while helices 3, 5, 7, and 9 participate in the formation of the ligand-binding groove. The most conserved residues are located within the groove, while the outer surface of the molecule is less conserved [46, 47] (Fig. 1b).

The C-terminal region of plant 14-3-3 proteins contains a number of important functional elements, including a conserved nuclear export signal (NES) inherent in mammalian, plant, and fungal 14-3-3 proteins [48, 49]. The role of NES in the functioning of 14-3-3 proteins was shown in *Schizosaccharomyces pombe* yeast. Mutation in the NES sequence of Rad24 (14-3-3 family protein) abolished the nuclear export of phosphorylated Cdc25 which normally signals DNA damage [50]. Similarly, 14-3-3 was found to regulate the signaling associated with DNA damage through COP1 (constitutive photomorphogenic 1) protein, a ubiquitin ligase of p53 protein. In the case of DNA damage, COP1 undergoes phosphorylation, interacts with 14-3-3 σ, and is exported from the nucleus [51]. Mutations in the NES of 14-3-3 σ prevented the export of COP1 induced by DNA damage [51]. It is believed that the NES sequence is essential for the regulation of transcription factors by 14-3-3 proteins through their retention in the cytoplasm (cytoplasmic sequestering) and blockade of their entry to the nucleus. Thus, the binding of 14-3-3 ε to the mitosis-regulating protein Cdc25 from the clawed frog (*Xenopus*) prevented Cdc25 from entering the nucleus and resulted in its cytoplasmic localization [52].

The C-terminus of 14-3-3 is disordered, non-conserved, and has different length and amino acid sequence in different 14-3-3 isoforms and homologs from different organisms [53]. Based on the secondary structure prediction by bioinformatics methods and data of circular dichroism analysis of the C-terminal peptide of 14-3-3 ω from *A. thaliana*, Shen et al. [54] suggested that the C-terminal region of this protein might contain a tenth α-helix. The question on the existence of this additional C-terminal α-helix remains unresolved, since the tenth α-helix is absent from any resolved spatial structure of plant 14-3-3.

It was suggested that the C-terminus of mammalian 14-3-3 isoforms has an autoinhibitory function. Thus, 14-3-3 ζ lacking the C-terminus exhibited a higher affinity for its protein partners Raf-1 and Bad [55]. Using the FRET method, Silhan et al. [56] demonstrated that the C-terminus is located in the amphipathic groove and is displaced from it upon phosphopeptide binding [56]. The autoinhibitory function of the C-terminus in plant 14-3-3 proteins has been proposed in several studies. Shen et al. [54] showed that the 14-3-3 ω isoform truncated at the C-terminus had a higher inhibitory activity towards its partner protein nitrate reductase (NR), thus indirectly indicating the autoinhibitory role of the C-terminus [54]. However, Athwal et al. [57] found that the C-terminal truncation of 14-3-3 ω, on the opposite, significantly reduced the inhibitory capacity of this protein towards NR [57]. The autoinhibitory role of the C-terminus was also observed in the study of the plasma membrane H^+-ATPase (AHA1), a well-known partner protein of 14-3-3. Thus, 14-3-3 ω and ε truncated at the C-terminus activated AHA1 more efficiently than the wild-type isoforms [58]. Deletion of the C-terminus from the T14-3c isoform of tobacco (*Nicotiana tabacum*) increased the protein affinity to sucrose phosphate synthase (SPS) [59].

Plant 14-3-3 proteins can bind divalent Ca^{2+} and Mg^{2+} ions in a region close to the C-terminus. The binding of calcium and magnesium ions to 14-3-3 and their effect on the protein structure have been confirmed by various experimental approaches. The tryptic cleavage patterns of 14-3-3 ω in the presence and absence of Ca^{2+} were different, suggesting conformational rearrangements induced by the binding of calcium ions [60]. Incubation with radioactive isotope $^{45}Ca^{2+}$ showed that unlike bovine serum albumin, membrane-immobilized 14-3-3 ω was able to bind Ca^{2+} ions [60]. Equilibrium dialysis experiments demonstrated that 14-3-3 ω bound calcium at a ratio of one Ca^{2+} ion per one 14-3-3 monomer [60]. The ability of 14-3-3 ω to bind multiply charged cations was shown using

terbium (Tb^{3+})-induced fluorescence, as the appearance of characteristic fluorescence peaks indicated Tb^{3+} binding [61]. The binding of Ca^{2+} to the 14-3-3 κ and λ isoforms was demonstrated by thermophoresis [62]. It was suggested that the ion-binding site of 14-3-3 is located in the loop between the helices 8 and 9 [57, 60]. Using manual comparison of sequences of *A. thaliana* 14-3-3 ω and calmodulin, Lu et al. [60] found a similarity between individual amino acid residues in 14-3-3 ω with the residues in the EF-hand domain of calmodulin [60]. However, automated bioinformatics search for *A. thaliana* proteins containing EF-hands performed by Day et al. revealed no EF-hand domains in 14-3-3 proteins [63]. Using the surface plasmon resonance method, it was shown that increasing concentrations of Ca^{2+} and Mg^{2+} promoted the binding of 10 tested 14-3-3 isoforms from *A. thaliana* (χ, κ, λ, ν, ω, φ, ψ, υ, ε, and μ) with synthetic phosphopeptides from the partner proteins nitrate reductase (NR2) and plasma membrane H^+-ATPase (AHA2), which indirectly indicates that the binding of these divalent ions by 14-3-3 can strengthen its interaction with the partner proteins [64]. It was also noted that the presence of Ca^{2+} and Mg^{2+} in the reaction buffer increased the inhibitory activity of 14-3-3 ω against NR [57] and its ability to bind phosphopeptides [61]. On the contrary, Ca^{2+} ions decreased the inhibitory function of 14-3-3 λ and κ toward the partner protein SOS2 kinase [62]. It should be emphasized that at the moment, there is no direct experimental evidence of divalent cation binding in the region between the helices 8 and 9 obtained based on the spatial structure of 14-3-3. Also, since 14-3-3 proteins are acidic, nonspecific binding of cations cannot be excluded. Therefore, the questions about specific binding of Ca^{2+} and Mg^{2+}, as well as the identity and location of the cation-binding site in 14-3-3 remain unresolved and controversial.

The properties of 14-3-3 dimers have been studied in most detail for mammalian 14-3-3 isoforms. Thus, the dissociation constant (*K*d) of human 14-3-3 ζ dimer measured by several methods was found to be ~5 nM [65], indicating very strong binding. Human 14-3-3 isoforms have also been studied for the monomer preferences upon dimerization. It was found that 14-3-3 ε tends to form heterodimers with other isoforms [66, 67], while 14-3-3 σ preferentially homodimerizes [68, 69]. A possible explanation lies in the number and representation of certain amino acid residues and chemical contacts in the region of dimerization. The 14-3-3 σ monomers are linked in the homodimer by three pairs of salt bridges (three symmetrical contacts: Arg19–Glu91, Asp21–Lys87, Lys9–Glu83) and several additional contacts, which provides a high stability of the homodimer. According to the hypothesis of Yang et al. [67], 14-3-3 ε monomers form only one pair of salt bridges in the homodimer (two symmetrical contacts of Arg19 of one monomer and Glu92 of the other monomer), while heterodimerization with other 14-3-3 isoforms involves formation of an additional salt bridge in 14-3-3 ε [e.g., Glu92(ε)–Arg18(ζ) upon interaction with 14-3-3 ζ], which makes 14-3-3 ε heterodimerization more preferable [67, 69]. Interestingly, in some cases, 14-3-3 heterodimers can bind to partner proteins, as established from the spatial structure of the human PEAK3 pseudokinase complex with the 14-3-3 ε/β heterodimer [70]. Heterodimers containing human 14-3-3 ε can efficiently form *in vitro* under native conditions via exchange of different homodimer subunits [71, 72], but the functions of such heterodimers *in vivo* remain a subject of debate.

Plant 14-3-3 are likely capable of heterodimerization as well. 14-3-3 isoforms from *A. thaliana* were found to form χ + φ/ω/ψ, ω + φ/υ/ψ, φ + υ/ψ, and ψ + υ heterodimers [73]; the ω isoform was shown to heterodimerize with κ and λ isoforms [74]. However, in the experiments on subunit heterodimerization, the mixed 14-3-3 isoforms were first denatured in the presence of guanidine chloride [73] or deoxycholate [74] and then subjected to renaturation, followed by analysis of the homo- and heterodimers formed. These experimental conditions are very far from the native ones and only indirectly suggested the possibility of formation of certain dimers in plant cells. Yet, there is evidence that 14-3-3 heterodimerization can occur *in vivo*, although the isoforms involved in this process remain unknown [73, 75, 76]. It is important to note that heterodimerization of plant 14-3-3 isoforms has been studied only for proteins from the non-epsilon phylogenetic group, while the data on heterodimerization of epsilon group isoforms are lacking. At the same time, an interesting information was obtained by using the yeast two-hybrid method to study pairwise dimerization of six 14-3-3 isoforms from the cotton plant (*Gossypium hirsutum*), three of which were from the epsilon group and three – from the non-epsilon group. The studied isoforms formed only certain heterodimers (Gh14-3-3L and Gh14-3-3e, Gh14-3-3L and Gh14-3-3g, Gh14-3-3a and Gh14-3-3e, Gh14-3-3a and Gh14-3-3g, Gh14-3-3g and Gh14-3-3h), but not homodimers [77]. Verification of these results by direct biochemical methods will be of great interest.

14-3-3 proteins can be phosphorylated, and this modification can influence dimer dissociation. Phosphorylation of Ser58 in the dimer interface of human 14-3-3 ζ resulted in dimer destabilization and dissociation [78]. In plant 14-3-3 proteins, residues homologous to mammalian Ser58 can also be phosphorylated *in vivo*. The phosphomimetic modification of homologous Ser62 residue in 14-3-3 ω facilitated dimer dissociation [79, 80]; this effect was even more pronounced upon introduction of two phosphomimetic substitutions simultaneously (at Ser62 and Ser67) [79].

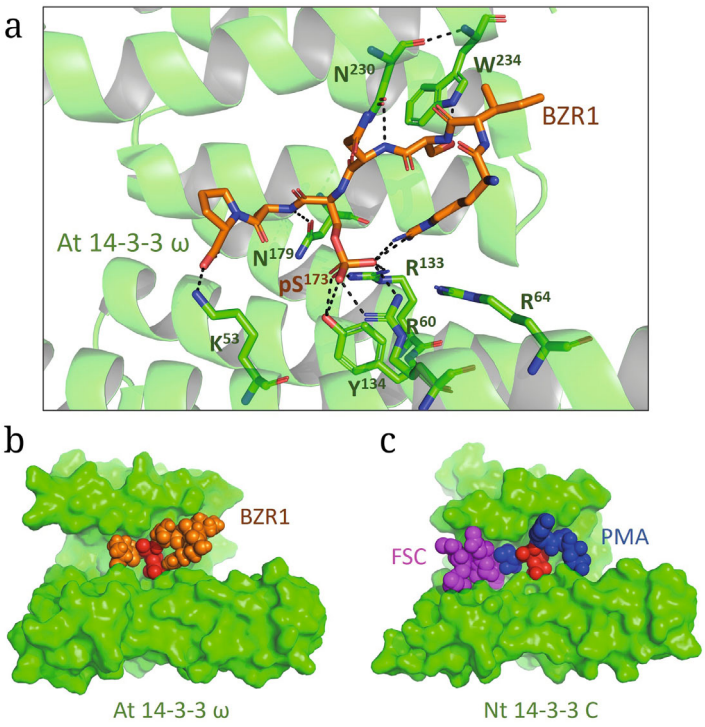

Fig. 2. Interaction of 14-3-3 proteins with phosphopeptides. a) Binding of phosphopeptide from BZR1 transcription factor (motif II) in the amphipathic groove of 14-3-3 ω from *A. thaliana* (PDB ID: 8QTF [26]). Amino acid residues forming polar contacts (black dashed line) with the phosphate group and peptide backbone are shown. b) Interaction of 14-3-3 with the BZR1 phosphopeptide in the absence of fusicoccin (motif II; PDB ID: 8QTF [26]); orange, phosphopeptide; red, phosphoserine residue. c) Interaction of 14-3-3 with the PMA2a phosphopeptide and fusicoccin (motif III; PDB ID: 1O9F [88]); blue, phosphopeptide; red, phosphothreonine residue; pink, fusicoccin molecule. Images were created with the PyMol program.

The phosphomimetic substitution of Ser62 in 14-3-3 ω disrupted protein heterodimerization preferences and decreased 14-3-3 affinity for its ligands (peptide difopein and N-terminal fragment of NR) [74]. Interestingly, phosphorylation of 14-3-3 χ at Ser72 (analog of Ser67 in 14-3-3 ω) decreased its inhibitory activity toward NR [81]. Phosphorylation of residues homologous to Ser58 in plant 14-3-3 proteins was found to yield dissociation of calcium-dependent protein kinase 3 (CPK3) from 14-3-3 and the kinase degradation [82]. Therefore, phosphorylation of 14-3-3 proteins at the dimer interface can significantly alter the biological functions of these proteins by shifting the dimer/monomer equilibrium. Plant 14-3-3 proteins contain other phosphorylation sites [46, 83], but the consequences of their modification and its effect on the structure and functions of 14-3-3 proteins have been studied insufficiently.

Principles of 14-3-3 binding to partner proteins. The binding partners of 14-3-3 proteins are typically phosphorylated at residues located in structurally disordered regions [84]. Based on numerous experimental data accumulated through the years of studies, it was found that 14-3-3 proteins bind phosphoserine (pS) or phosphothreonine (pT) residues in a specific amino acid context (consensus motif), and only in very rare cases interact with nonphosphorylated partners [85]. There are three types of 14-3-3-binding phosphorylated motifs: type I – RXXp(S/T)X(P/G), type II – RX(Y/F)Xp(S/T)X(P/G), and type III (C-terminal) – p(S/T)X_{0-2}–COOH, where p(S/T) is phosphoserine/phosphothreonine and X is any amino acid residue [48, 86] (Fig. 1c). There are data indicating that motif I in 14-3-3 partner proteins in plants can be slightly different. According to Johnson et al. [87], who conducted bioinformatics analysis of 14-3-3 recognition sequences in partner proteins, in many cases, plant motif I is extended at the N-terminus: LX(R/K)SX(pS/pT)XP [87]. The authors associated this feature with the fact that many light-dependent processes in plants are regulated by specific protein kinases that require the presence of a leucine residue in the beginning of motif I. However, the number of analyzed 14-3-3 recognition sites in plants was much smaller than for mammalian proteins (14 plant sequences vs. 201 mammalian sequences) [87], so that the validity of extrapolating these findings to all partners of plant 14-3-3 proteins is questionable.

The phosphopeptide binds along its entire length in the amphipathic groove via polar contacts and hydrophobic interactions. The main residues of 14-3-3 that form polar contacts with the phosphopeptide

backbone and phosphate group are Lys53, Arg60, Arg64, Arg133, Tyr134, Asn179, Asn230, and Trp234, which are highly conserved in plant and animal 14-3-3 proteins [88] (Fig. 2a; residue numbering for *Arabidopsis* 14-3-3 ω).

14-3-3 isoforms have different affinity for the partner protein phosphopeptides. Assessment of all 7 human 14-3-3 isoforms for their affinities to phosphopeptides derived from CTFR [89], USP8 [90], LRRK2 [91], etc., has shown that γ and η had the highest affinities, while σ and ε had the lowest affinities, and the remaining isoforms had intermediate affinities for the tested phosphopeptides. The affinity of 14-3-3 isoforms for the phosphopeptides from RSK1, HSPB6, and papillomavirus E6 protein decreased in the following order: γ>η>ζ>τ>β>ε>σ. The same hierarchy in the 14-3-3 isoform affinity was observed for large and diverse samples of phosphopeptides from various partner proteins [92]. It is quite likely that plant 14-3-3 proteins display a similar hierarchy of affinities for phosphorylated peptides.

Using the surface plasmon resonance method, it was demonstrated that 9 (out of 13 known) 14-3-3 isoforms from *A. thaliana* exhibited different affinities for the phosphorylated C-terminal peptide of the plasma membrane proton ATPase [47] that decreased in the following order: φ>χ>ν>ψ>υ>ε>ω>κ>λ. However, these data contradict the results obtained in the study on the effect of *A. thaliana* 14-3-3 isoforms (7 out 13) on the activity of the same plasma membrane ATPase [58], which decreased in the order: χ>ω>κ>λ>μ>ε>ο. The differences in the activity of several 14-3-3 isoforms from *A. thaliana* toward spinach leaf NR was demonstrated in [93]; in this case, the effect decreased in the following order: ω>φ>χ>ν [93]. According to Lambeck et al. [94], who studied the influence of 14-3-3 isoforms from *A. thaliana* on the NR activity, the inhibitory effect decreased in the following order: ω>λ>κ>ν>ψ>χ≫ε (the effect of 14-3-3 ε was so insignificant that the authors were unable to determine the half-inhibition constant) [94]. In a recent study, 7 isoforms of *A. thaliana* were examined (μ, ε, and ο from the epsilon group; λ, ν, ψ, and φ from the non-epsilon group) for their binding with the phosphopeptide from the FD protein (transcription factor and component of the florigen activation complex that triggers flowering). According to the data obtained, the affinity decreased as follows: ψ>μ>λ>ν>φ>ο>ε [95]. Based on the above, the data on the affinity of 14-3-3 proteins to the phosphopeptides of partner proteins are scarce and contradictory. No correlation was found between the affinity of a 14-3-3 isoform to the partner proteins and isoform attribution to the epsilon or non-epsilon group. Apparently, more systematic studies using all rather than a few randomly selected isoforms are required to make confident statements about the existence and nature of the hierarchy of the binding affinity of plant 14-3-3 isoforms. It should be noted that even though such hierarchy has been proven for 7 relatively conserved human 14-3-3 isoforms [92], its nature remains unclear, especially given that all 7 isoforms have the same structure of the phosphorylated residue-binding pocket in the amphipathic groove. This suggests that the binding strength may be influenced by currently unknown allosteric or long-range effects.

In addition to interactions at the primary binding site located in the amphipathic groove, the partner proteins can form contacts with other 14-3-3 regions, as it was observed for the florigen activating complex Hd3a [96] (vide infra). Since the outer regions of 14-3-3 dimers are the least conserved, their interactions may be specific to particular isoforms.

MAIN FUNCTIONS OF PLANT 14-3-3 PROTEINS

General principles of functioning of 14-3-3 proteins. Similar to their animal and fungal orthologs, plant 14-3-3 proteins lack enzymatic activity and function by directly interacting with phosphorylated partner proteins. As a rule, this interaction alters the activity of the partner protein, thus affecting its biological function. The mechanisms leading to such changes can be divided into the following categories.

Enzyme inhibition. In some cases, the binding of 14-3-3 protein can lead to the enzyme inhibition. A classic example is NR, whose binding to 14-3-3 presumably causes conformational changes leading to the suppression of its enzymatic activity [97] (Fig. 3a). Interaction of 14-3-3 with SPS also results in the enzyme inhibition [98]. Unfortunately, the lack of structural data makes it difficult to decipher the mechanism behind the inhibitory effect of 14-3-3.

Enzyme activation. The binding of 14-3-3 to an enzyme can also lead to the increase in its catalytic activity. Thus, the binding of 14-3-3 to the plant H^+-ATPase PMA2 results in the removal of the autoinhibitory sequence from the catalytic site and enzyme activation [58]. The spatial structure of the 14-3-3 complex with a fragment of the plasma membrane ATPase was one of the first resolved 3D structures. It was found that the binding of three 14-3-3 dimers induced the formation of ATPase hexamers [99] (Fig. 3b). The interaction of 14-3-3 with the cytosolic enzyme glutamine synthase (GS1) also promoted enzyme activation [100].

Changes in protein location. The binding of 14-3-3 to a partner protein can alter the intracellular location of the latter. Thus, the interaction between phosphorylated transcription factors BZR1 (BRASSINAZOLE RESISTANT 1) and BES1 (BRI1 EMS SUPPRESSOR 1) involved in the intracellular signaling mediated by

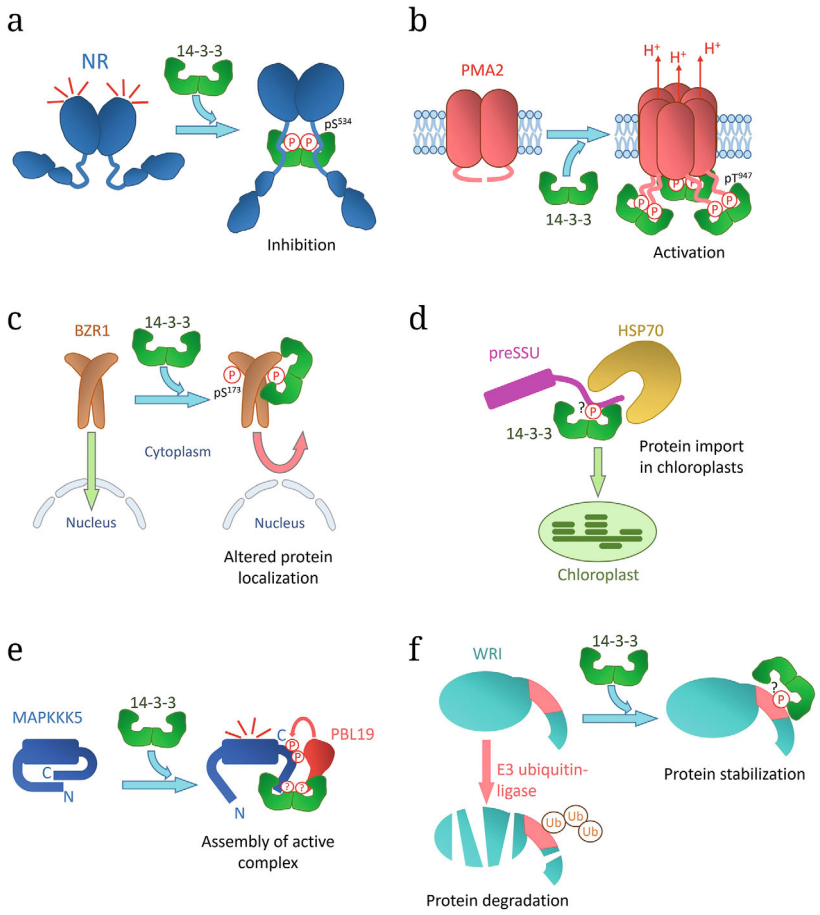

Fig. 3. Regulation of functional activity of partner proteins by plant 14-3-3 proteins. a) NR inhibition by 14-3-3 binding. b) Activation of plasma membrane H^+-ATPase PMA2 by 14-3-3 binding. c) Changes in the intracellular location (cytoplasmic retention) of the transcription factor BZR1 mediated by 14-3-3 proteins. d) Import of the precursor of Rubisco small subunit (preSSU) into chloroplasts with the assistance of 14-3-3 and HSP70. e) Phosphorylation and activation of protein kinase MAPKKK5 by protein kinase PBL19 in complex with 14-3-3. f) Inhibition of degradation of the transcription factor WRI after binding to 14-3-3.

brassinosteroids (plant hormones) prevents the entry of these factors to the nucleus and further regulation of gene expression [101, 102] (Fig. 3c). Other transcription factors, such as RSG (Repression of Shoot Growth) [103] and PIF7 (Phytochrome-Interacting Factor 7) [104], also appear to be regulated through cytoplasmic retention.

Protein transport into chloroplasts and mitochondria. Participation of 14-3-3 in protein transport into chloroplasts has been suggested already in the early studies. It was shown that a complex of 14-3-3 with HSP70 promoted the import of Rubisco small subunit precursor (preSSU) to chloroplasts [105] (Fig. 3d). Also, 14-3-3 was found to interact with the leader sequence of *Arabidopsis* photosystem I N-subunit (PSI-N) [106]. According to [107], 14-3-3 promoted the import of at least 11 chloroplast proteins into chloroplasts. 14-3-3 proteins also participate in protein transport into mitochondria. It is known that the signal sequences of many proteins imported into mitochondria are phosphorylated [108]. In this case, the role of 14-3-3 might be negative: the binding of MORF3 (Multiple Organellar RNA editing Factor) phosphorylated at the signal sequence with a complex of 14-3-3 and cytosolic HSP70 slowed down the import of MORF3 into mitochondria [109]. The details of mechanisms by which 14-3-3 affects the transport of proteins into double-membrane organelles remain unknown.

Assembly of active protein complexes. 14-3-3 proteins participate in the assembly of functional complexes, e.g., the transcriptional complex with VP1 (Viviparus1) and EmBP1 (*Em* promoter binding protein) that activates expression of the *Em* gene [110]. Formation of complexes with 14-3-3 isoforms can lead to the protein activation, as was demonstrated for the activation of MAPKKK5 by a complex of PBL19 kinase with 14-3-3 λ [111] (Fig. 3e). 14-3-3 κ and λ interact with the transcription factor PIF3 (Phytochrome-Interacting Factor 3) and photoactivated phytochrome phyB and stabilize the complex of these two proteins

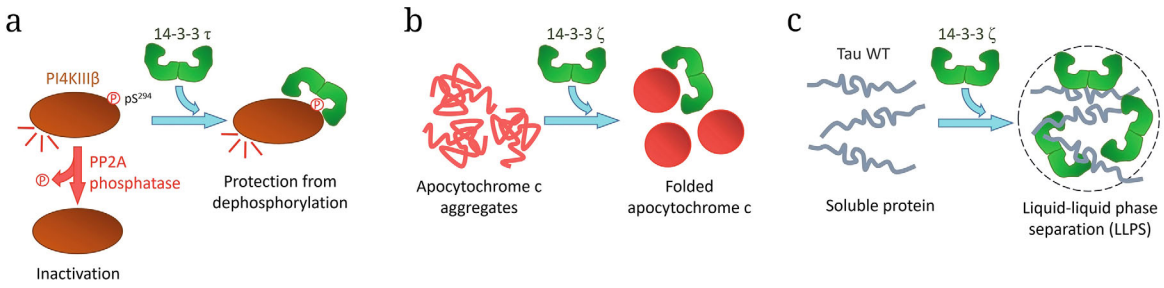

Fig. 4. Mechanisms by which animal 14-3-3 proteins regulate the functions of partner proteins (no similar activities have been identified for plant 14-3-3 proteins). a) Protection of PI4KIIIβ from dephosphorylation by 14-3-3 τ binding. b) Disaggregation of apocytochrome *c* by the chaperone-like activity of 14-3-3 ζ. c) Liquid–liquid phase separation (LLPS) after 14-3-3 ζ binding to the wild-type tau protein.

in the nucleus, which ultimately results in PIF3 degradation and initiation of photomorphogenesis processes [112].

Effect on protein half-life. 14-3-3 proteins can modulate stability of partner proteins by influencing their susceptibility to intracellular degradation. For example, the binding of 14-3-3 to the key enzyme of ethylene biosynthesis 1-aminocyclopropane-1-carboxylate synthase (ACS) increases stability of this enzyme, while 14-3-3 binding to the components of E3 ubiquitin ligase ETO1/EOL, on the contrary, promotes degradation of these proteins [113]. Associated with the transcription factor WRI1, 14-3-3 prevents its degradation by shielding the binding site for E3 ubiquitin ligase [114] (Fig. 3f).

Suppression of dephosphorylation. The binding of 14-3-3 can prevent dephosphorylation of partner proteins by shielding the phosphate group from phosphatases, as demonstrated in studies on human 14-3-3 isoforms. For example, the interaction of 14-3-3 τ with phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ) increased the content of phosphorylated active PI4KIIIβ [115] (Fig. 4a). The binding of 14-3-3 prevented dephosphorylation and degradation of the transcription factor FoxO3 involved in the control of cell proliferation and apoptosis [116]. No inhibitors of dephosphorylation have been found among plant 14-3-3 proteins yet; however, given their structural and functional similarity to mammalian 14-3-3 proteins, this function can be expected in plant proteins as well.

Chaperone-like activity. Mammalian 14-3-3 proteins exhibit chaperone-like activity, i.e., prevent aggregation of substrate proteins in an ATP-independent manner. Mammalian 14-3-3 ζ was found to prevent aggregation of insulin, alcohol dehydrogenase, and phosphorylase kinase *in vitro* [117]; 14-3-3 ζ from *Drosophila* prevented aggregation of citrate synthase, as well as prevented aggregation and even promoted disaggregation of apocytochrome *c* aggregates *in vivo* [118] (Fig. 4b). Since this activity does not depend on phosphorylation of partner proteins, it can be considered as a "moonlighting" function of 14-3-3 [53]. There are reasons to believe that plant 14-3-3 proteins also possess chaperone-like activity, especially considering a potentially greater susceptibility of plant cells to environmental changes (e.g., in ambient temperature).

Regulation of liquid-liquid phase separation (LLPS). Recent studies have demonstrated that mammalian 14-3-3 proteins can modulate LLPS by interacting with partner proteins. Thus, it was shown that 14-3-3 ζ binds to unmodified tau protein, promoting formation of droplets of the tau protein phase during LLPS and stabilizing them [119] (Fig. 4c). It cannot be ruled out that plant 14-3-3 proteins perform a similar function in LLPS in plants.

14-3-3 proteins as regulators of plant metabolism. In plants, 14-3-3 proteins regulate primary metabolism by interacting with and controlling the activity of key enzymes of nitrogen, carbohydrate, and sulfur metabolism.

Regulation of nitrogen metabolism. The most studied protein partner of 14-3-3 in plants is NR, an enzyme involved in nitrogen metabolism. It is a cytoplasmic protein that catalyzes the first step of nitrogen assimilation in plants, namely, NADH-dependent conversion of nitrate to nitrite (Fig. 5). Nitrite is reduced in chloroplasts to ammonium ion, which is then incorporated into amino acids [120, 121]. NR is a large protein; the molecular weight of its dimer is ~200 kDa [122]. The N-terminal domain of NR contains molybdenum as a cofactor and is followed by the cofactor-free dimerization domain, central domain with the heme *b5*, and C-terminal domain that contains bound FAD and has the NADH-binding site [122] (Fig. 5). Because of its important biological role and dependence on reducing equivalents (which are actively supplied during the daytime), NR is tightly regulated by many factors, including 14-3-3 proteins [121]. The enzyme is phosphorylated at a serine residue located approximately in the middle of its polypeptide chain (Ser543 in spinach NR). The sequence containing this residue corresponds to the 14-3-3 recognition motif I [61]. Indeed, this motif is recognized by 14-3-3 proteins [61], leading to NR inhibition [93, 94, 123]. The binding of 14-3-3 blocks

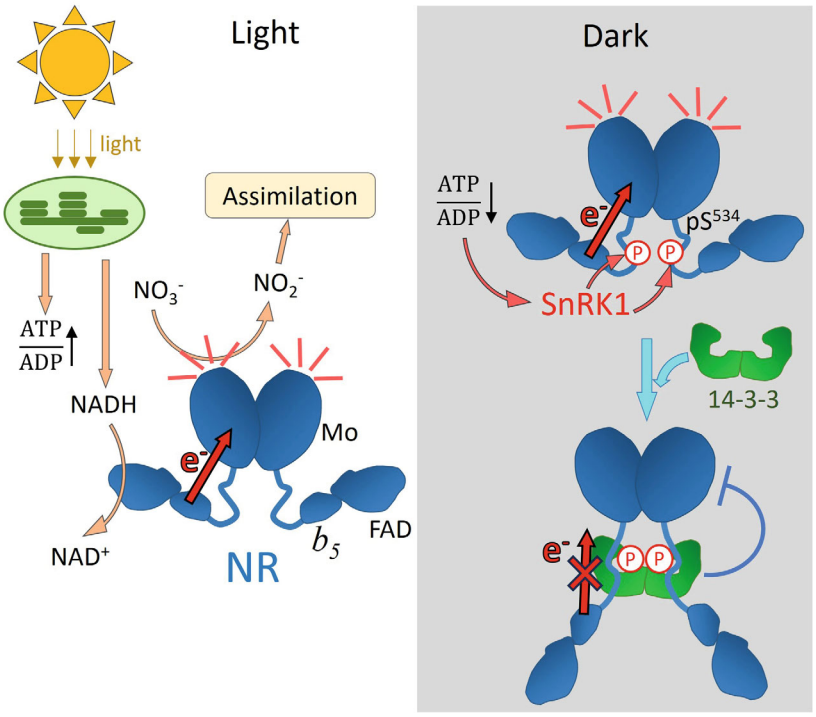

Fig. 5. Regulation of nitrate reductase (NR) activity by 14-3-3 proteins. Illumination induces formation of reducing and energy equivalents via photosynthesis. Active NR reduces nitrate ions to nitrites in the cytoplasm. During catalysis, electrons are transferred from NADH to the FAD-containing domain (FAD) and then to the heme *b5*-containing domain (b5) and molybdenum-containing domain (Mo), where nitrate is reduced to nitrite. Nitrite is reduced to ammonium, which is then incorporated into organic compounds (e.g., amino acids). In the dark, the amount of reducing equivalents decreases and nitrate reduction stops, so NR should be inhibited. A decrease in the ATP/ADP ratio leads to the SnRK1 activation and phosphorylation of NR at the 14-3-3 recognition motif located between the Mo and b5 domains (at Ser543 in spinach NR). The binding of 14-3-3 to this site causes conformational changes in the enzyme, resulting in the spatial separation of the Mo and b5 domains, which hinders electron transfer between them and leads to NR inactivation.

electron transfer from the heme to the molybdenum cofactor [97]. Apparently, the binding of 14-3-3 in the region between the two cofactors causes conformational rearrangements that move the cofactors away from each other and make them to adopt a conformation that hinders electron transport [97] (Fig. 5). Chi et al. [124] provided a more detailed explanation of this proposed mechanism [124]. They showed that in NR from *A. thaliana*, 14-3-3 binds not only to motif I in the proximity of phosphorylated serine, but also to the conserved acidic N-terminal motif and that complete inhibition of NR requires simultaneous 14-3-3 binding to both sites [124]. Interestingly, the binding to the acidic N-terminal motif probably occurs outside the phosphopeptide-binding groove of 14-3-3 [124]. The key serine residue in the 14-3-3-recognizing site of NR is phosphorylated by the protein kinase SnRK1 (SNF1-related kinase 1) [125]. This enzyme is known to suppress anabolic and to activate catabolic processes upon the deficit of energy equivalents in cells [126, 127]. Thus, phosphorylation of NR by SnRK1 under the energy deficit conditions (in the dark) promotes the binding of 14-3-3 and conformational rearrangements leading to the enzyme inhibition. Despite an overall elegance of the proposed mechanism, its details remain poorly understood due to the lack of information on the structure of the NR complex with 14-3-3 protein.

Cytosolic GS1, a downstream enzyme in the nitrogen assimilation pathway, is also regulated by 14-3-3 proteins. GS1 converts glutamic acid to glutamine, resulting in the ammonium incorporation into organic compounds (Fig. 6a). Finnemann et al. [100] showed that the binding of 14-3-3 to phosphorylated GS1 increased its catalytic activity in aging rapeseed (*Brassica napus*) leaves [100]. The phosphorylated residue in GS1, as well as kinase and phosphatase involved in the enzyme regulation, remain unknown. Activation of GS1 in aging leaves is necessary for the nitrogen removal from tissues in the form of organic compounds [100]. It was also shown that 14-3-3 interacts with phosphorylated GS1 from *Chlamydomonas reinhardtii* [128]. In this case, GS1 is presumably phosphorylated by the Ca^{2+}/calmodulin-dependent protein kinase, however, neither phosphorylation, nor the binding of 14-3-3 had any effect on the GS1 activity [128]. Glutamine synthetase GS2 is a GS isoform located in chloroplasts. Western blotting of native GS2 oligomers isolated from tomato chloroplasts demonstrated that

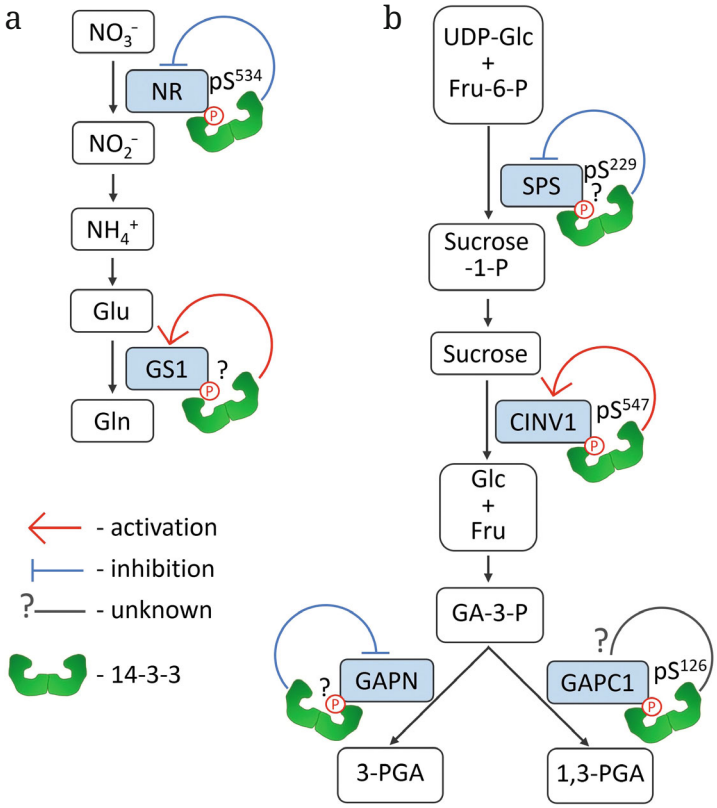

Fig. 6. Hypothetical scheme of plant metabolism regulation by 14-3-3 proteins. The scheme is based on the most reliable data (in the opinion of the authors of this review) on the effects of 14-3-3 on the functional activity of partner proteins. a) Regulation of nitrogen metabolism. b) Regulation of carbohydrate metabolism. Designations: Glc, glucose; Fru, fructose; UDP-Glc, UDP-glucose; Fru-6-P, fructose 6-phosphate; Sucrose-1-P, sucrose-1-phosphate; GA-3-P, glyceraldehyde 3-phosphate; 3-PGA, 3-phosphoglycerate; 1,3-PGA, 1,3-bisphosphoglycerate; NR, nitrate reductase; GS1, glutamine synthetase 1; SPS, sucrose-phosphate synthase; CINV1, alkaline invertase 1; GAPN, non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase; GAPC1, cytosolic glyceraldehyde 3-phosphate dehydrogenase 1.

enzymatically active GS2 octamer contained 14-3-3 [129]. It was suggested that dissociation of 14-3-3 from the complex results in the loss of GS2 activity [129]. This is an unusual finding, since 14-3-3 typically localizes to the cytosol or the nucleus. The question of 14-3-3 presence in chloroplasts requires further investigation. A detailed study on the effect of glutamine synthetase phosphorylation at various residues on its interaction with 14-3-3 and enzymatic activity *in vitro* will provide better understanding of the regulation of this important enzyme.

Regulation of carbohydrate metabolism. 14-3-3 proteins regulate carbohydrate metabolism at various levels. Several interactome studies have identified dozens of carbohydrate metabolism enzymes interacting with 14-3-3 proteins [130-133].

SPS catalyzes conversion of fructose-6-phosphate and UDP-glucose into sucrose-1-phosphate, an immediate precursor of sucrose (Fig. 6b). SPS from spinach (*Spinacia oleracea*) is regulated by reversible phosphorylation at Ser158 that depends on the time of day and illumination [134]. In the dark, when there is no photosynthesis and the flow of metabolites decreases, SPS is phosphorylated to inhibit its activity. Exposure to light induces enzyme dephosphorylation and its activation [134, 135]. It was shown that SPS, like NR, is phosphorylated by SnRK1 (metabolic regulator that inhibits anabolic reactions) at Ser158 [125]. Phosphorylated SPS binds to 14-3-3 [98, 136]. Using surface plasmon resonance, Toroser et al. [98] showed that 14-3-3 binds spinach SPS at Ser229 residue. However, further analysis of 14-3-3 affinity to phosphopeptides demonstrated that none of the 7 examined *A. thaliana* 14-3-3 isoforms bound *A. thaliana* SPS phosphopeptide homologous to the Ser229-containing motif from the spinach SPS; 14-3-3 instead interacted with *A. thaliana* SPS phosphopeptide homologous to the Ser158-containing fragment of the spinach enzyme [58]. The studies using site-directed mutagenesis and yeast two-hybrid assay failed to confirm the binding of 14-3-3 to tobacco SPS at the phosphorylated site homologous to Ser229 in spinach SPS [59]. Moreover, Ser229 is less conserved among SPS enzymes from different species than Ser158 [137]. These data cast doubts on the identity of 14-3-3-binding site characterized by Toroser et al. [98]. It was also shown that 14-3-3 binding

prevents SPS degradation *in vitro* [138]. The data on the effect of 14-3-3 on the SPS activity are controversial. According to Toroser et al., 14-3-3 binding inhibited SPS [98]. However, Moorhead et al. [136] showed that the phosphopeptide from Raf-1 (mammalian partner protein of 14-3-3) exhibited a weak inhibitory effect toward SPS, which may indicate activation of SPS by 14-3-3 proteins [136]. Unfortunately, conflicting data and lack of information on the structure of 14-3-3 complexes with SPS raise many questions that require further research.

Alkaline/neutral invertase CINV1 catalyzes the breakdown of sucrose to glucose and fructose (Fig. 6b). Using a yeast two-hybrid system, it was demonstrated that CINV1 from *A. thaliana* binds to 14-3-3 isoforms ε, κ, λ, υ, φ, χ, ψ, and ω, but not to μ [139]. The binding is mediated by Ser547, which can be phosphorylated by calcium-dependent protein kinases [139], and results in CINV1 activation [139]. Unfortunately, the information on the structure of CINV1 complexes with 14-3-3 is lacking.

Another carbohydrate metabolism enzyme interacting with 14-3-3 proteins is non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPN) from wheat [140]. This enzyme catalyzes irreversible oxidation of glyceraldehyde 3-phosphate into 3-phosphoglycerate with the reduction of $NADP^+$ (Fig. 6b). It is believed that GAPN is especially important in providing non-photosynthetic tissues with NADPH [140, 141]. The studies on the effect of phosphopeptides on the activity of GAPN complex with 14-3-3 revealed that the interaction between these two proteins causes enzyme inhibition and a 3-fold decrease in the maximum rate of the GAPN-catalyzed reaction [140]. However, both the 14-3-3 recognition site and the kinase that phosphorylates it remain unknown.

14-3-3 proteins also interact with the cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPC1) from *A. thaliana* [138] (Fig. 6b). Most likely, the residue involved in the 14-3-3 binding is Ser126 in the KVVIpSEP sequence, which is similar to the type II recognition motif [58, 142], but the effect of this interaction remains unknown.

Plant 14-3-3 proteins can bind to and influence the functions of proteins regulating carbohydrate metabolism. Thus, 14-3-3 has been shown to bind *A. thaliana* trehalose phosphate synthase TPS5 [143], which catalyzes formation of trehalose 6-phosphate, a signaling molecule regulating carbohydrate metabolism. The binding is mediated by Ser22 and Thr49 residues located close to the protein N-terminus. In both cases, the recognition motifs for 14-3-3 are similar to type motifs, but differ from the canonical ones by the absence of proline residue at position +2 [58, 143]. *In vitro*, these residues are phosphorylated by SnRK1 [143]. Another important regulatory enzyme of carbohydrate metabolism, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (F2KP), also interacts with 14-3-3. F2KP synthesizes fructose-2,6-bisphosphate, a signaling metabolite which is believed to direct sugar metabolism toward the synthesis of either sucrose or starch [144]. Using pull-down assay, Kulma et al. [145] showed that *A. thaliana* F2KP specifically bound 14-3-3 in cell extracts, presumably through Ser220 and Ser303 residues [145]. However, no significant effect of 14-3-3 on the F2KP activity has been found *in vitro*, which might be due to poorly designed experimental conditions [145].

Many 14-3-3 interaction partners involved in glycolysis and other carbohydrate metabolism reactions have been identified in interactome studies. These include phosphoenolpyruvate carboxylase, glucose-6-phosphate dehydrogenase [133], phosphoglycerate isomerase, pyruvate kinase, transketolase, sucrose phosphate synthase [130], triosephosphate isomerase, enolase, and fructose-bisphosphate aldolase [132]. Unfortunately, this information has not yet been confirmed by direct studies of the interaction of these enzymes with 14-3-3 proteins, which could be an interesting area of future research.

Regulation of sulfur metabolism. Shin et al. [146] identified a number of enzymes of the sulfur assimilation pathway as 14-3-3 partners [146]. Among them, two enzymes of cysteine synthesis, o-acetylserine (thiol) lyase (OASTL) and serine acetyltransferase (SAT), have been investigated in most detail, and their interaction with 14-3-3 χ was confirmed by coimmunoprecipitation [146]. However, the details of 14-3-3 interaction with sulfur metabolism proteins remain enigmatic.

Therefore, there is a large body of evidence indicating that 14-3-3 proteins are involved in the regulation of key enzymes of nitrogen, carbohydrate, and sulfur metabolism in plants. However, a clear understanding of the global role of 14-3-3 in these important processes is lacking. Moreover, structural and functional details of 14-3-3 interaction with metabolic enzymes remain unknown, and reported experimental data are often contradictory.

Plant 14-3-3 proteins as regulators of membrane transport. 14-3-3 are soluble cytosolic proteins; however, they can regulate the activity of membrane transporters and enzymes by binding to them from the cytosolic side. In a number of studies, plant 14-3-3 proteins have been found in the proximity of the plasma membrane [37, 76, 81, 147].

Regulation of transport across the plasma membrane. One of the most well-characterized protein-protein interactions of plant 14-3-3 proteins is binding to the plasma membrane H^+-ATPase (PMA), or AHA (*Arabidopsis* H^+-ATPase/autoinhibited H^+-ATPase). PMA belongs to the family of P-type cation-transporting ATPases and transfers protons from the cell to the

extracellular medium with the expenditure of energy. It is responsible for the formation of the transmembrane proton gradient used as an energy source for the secondary active transport in plant cells [148]. The C-terminus of PMA contains the autoinhibitory sequence [149]. PMA binds 14-3-3 at the phosphorylated C-terminal motif YpTV-COOH (type III motif). The residue that undergoes phosphorylation in PMA2 from *Nicotiana plumbaginifolia* is Thr948 [150, 151]. This C-terminal motif is a part of the autoinhibitory domain [152]. The binding of 14-3-3 to PMA abolishes autoinhibition and activates the enzyme [58, 151] (Fig. 3b). Activated PMA oligomerizes with the formation of the active complex containing six PMA subunits and three 14-3-3 molecules, as was shown by a combination of X-ray crystallography and cryo-electron microscopy [99] (Fig. 3b; Fig. 7a). The activity of PMA provides a decrease in the apoplast pH and maintenance of the transmembrane proton gradient, which is necessary for normal plant growth and development, e.g., elongation of hypocotyl and roots [153], activity of stomatal cells [99, 154], and pollen tube growth [155]. The binding between 14-3-3 and PMA is a target of the pathogenic ascomycete fungus *Phomopsis amygdali* (*Fusicoccum amygdali*). This fungus synthesizes and secretes the toxin fusicoccin, which greatly increases the affinity of 14-3-3 for the type III motif in PMA by occupying a free space in the amphipathic groove of the 14-3-3 complex with the short C-terminal phosphopeptide [88] (Fig. 2, b and c). Fusicoccin itself binds rather weakly to 14-3-3 (dissociation constant *K*d, ~66 μM), and simultaneous presence of the phosphopeptide is important for increasing its binding strength [88, 156]. The mechanism of action of fusicoccin was elucidated after resolving the spatial structure of its complex with 14-3-3 and PMA2 phosphopeptide. This discovery has initiated an entire new direction in the studies of 14-3-3 proteins. Currently, the structures of plant 14-3-3 proteins most represented in the Protein Data Bank are those of *N. tabacum* 14-3-3 complexes with fragments of *N. plumbaginifolia* PMA2 (7 out of 19 structures: 3 complexes with the phosphopeptide [88, 157] and 4 complexes with a longer C-terminal fragment of PMA2 [99, 158, 159]) (Table 3). PMA activation leads to membrane hyperpolarization, opening of stomata, and uncontrolled transpiration [160]. The inability to regulate water exchange leads to a pathological phenotype with yellowing and drying leaves [160]. Notably, one of the first names of plant 14-3-3 was fusicoccin-binding protein (FCBP) due to the discovery of 14-3-3 in the fusicoccin-containing membrane-bound protein complex with PMA [8, 161-163]. Later, it has become clear that fusicoccin stabilizes not only the binding between 14-3-3 and PMA, but also other interactions of 14-3-3 with plant and animal partner proteins through the type III motifs [164, 165].

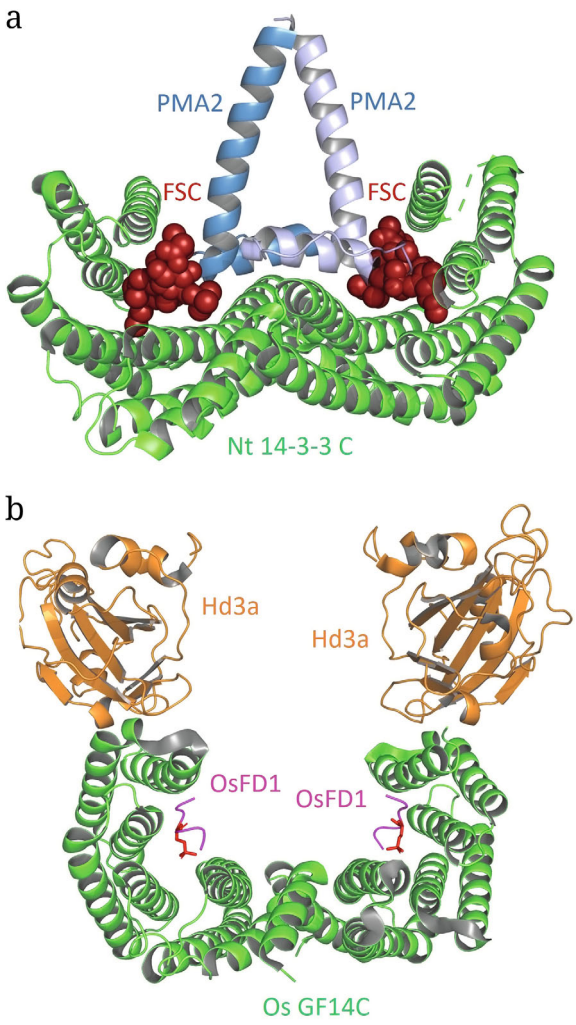

Fig. 7. Spatial structures of 14-3-3 complexes with partner proteins. a) Complex of 14-3-3 isoform C from *N. tabacum* (green) with two C-terminal fragments of PMA2 from *N. plumbaginifolia* (blue and light blue) and fusicoccin (red) (PDB ID: 2O98 [99]). b) Complex of 14-3-3 GF14C isoform from *O. sativa* (green) with florigen Hd3a (orange) and phosphopeptide from OsFD1 transcription factor (pink, phosphoserine residue is shown in red) (PDB ID: 3AXY) [96]. Images were created with PyMol.

Structural studies have identified a number of small molecules that can stabilize the interaction of 14-3-3 and PMA2, including cotylenin A [157], pyrrolinone derivative pyrrolidone 1 [158], dipeptide epibestatin [158], and pyrazole derivative [159]. Analysis of the obtained spatial structures of protein complexes with these compounds revealed that the sites of their binding to 14-3-3 were similar to that of fusicoccin (Table 3).

Despite generally recognized function of fusicoccin as a PMA activator, there is a growing body of evidence that this small molecule has other physiological effects, such as regulation of potassium ion currents, enzymes, phytohormones, and protein kinase

Table 3. Spatial structures* of complexes formed by plant 14-3-3 proteins (in reverse chronological order)

PDB ID	14-3-3 protein	Partner protein or ligand	Recognition motif type	Year released	References
8QTC	*A. thaliana* 14-3-3 ω	phosphopeptide from *A. thaliana* transcription factor BZR1	II	2023	[26]
8QTF	*A. thaliana* 14-3-3 ω	phosphopeptide from *A. thaliana* transcription factor BZR1	II		
8QT5	*A. thaliana* 14-3-3 λ	phosphopeptide from *A. thaliana* transcription factor BZR1	II		
8QTT	*A. thaliana* 14-3-3 ω	phosphopeptide from *A. thaliana* BRI1 kinase inhibitor	–		
8HEW	*S. tuberosum* St14f	phosphopeptide from *S. tuberosum* StFDL1	I	2023	[182]
7XBQ	*S. tuberosum* St14f	–	–	2022	[183]
5NWI	*N. tabacum* 14-3-3 C	C-terminal peptide from *A. thaliana* KAT1 potassium channel	III	2017	[165]
5NWJ	*N. tabacum* 14-3-3 C	C-terminal peptide from *A. thaliana* KAT1 potassium channel			
5NWK	*N. tabacum* 14-3-3 C	C-terminal peptide from *A. thaliana* KAT1 potassium channel and fusicoccin			
4DX0	*N. tabacum* 14-3-3 E	C-terminal peptide from *N. plumbaginifolia* ATPase PMA2 and pyrazole derivative	III	2012	[159]
3AXY	*O. sativa* GF14-C	*O. sativa* florigen Hd3a, peptide from *O. sativa* transcription factor OsFD1	I	2011	[96]
3M51	*N. tabacum* 14-3-3 C	C-terminal peptide from *N. plumbaginifolia* ATPase PMA2 and pyrrolidone1	III	2010	[158]
3M50	*N. tabacum* 14-3-3 C	C-terminal peptide from *N. plumbaginifolia* ATPase PMA2 and epibestatin			
3E6Y	*N. tabacum* 14-3-3 C	C-terminal peptide from *N. plumbaginifolia* ATPase PMA2 and cotylenin A	III	2009	[157]
2O98	*N. tabacum* 14-3-3 C	C-terminal peptide from *N. plumbaginifolia* ATPase PMA2 and fusicoccin	III	2007	[99]
1O9E	*N. tabacum* 14-3-3 C	fusicoccin	-	2003	[88]
1O9C	*N. tabacum* 14-3-3 C	–	-		
1O9D	*N. tabacum* 14-3-3 C	C-terminal peptide from *N. plumbaginifolia* ATPase PMA2	III		
1O9F	*N. tabacum* 14-3-3 C	C-terminal peptide from *N. plumbaginifolia* ATPase PMA2 and fusicoccin	III		

Note. * As of July 2024.

signaling cascades. It was suggested that plants have an endogenous molecule that has a similar function as fusicoccin and triggers a coordinated physiological response involving a number of proteins, i.e., acts as a phytohormone [166]. Hence, the physiological activity of fusicoccin remains unclear. It is possible that new research directions will elucidate it and help to describe it in detail.

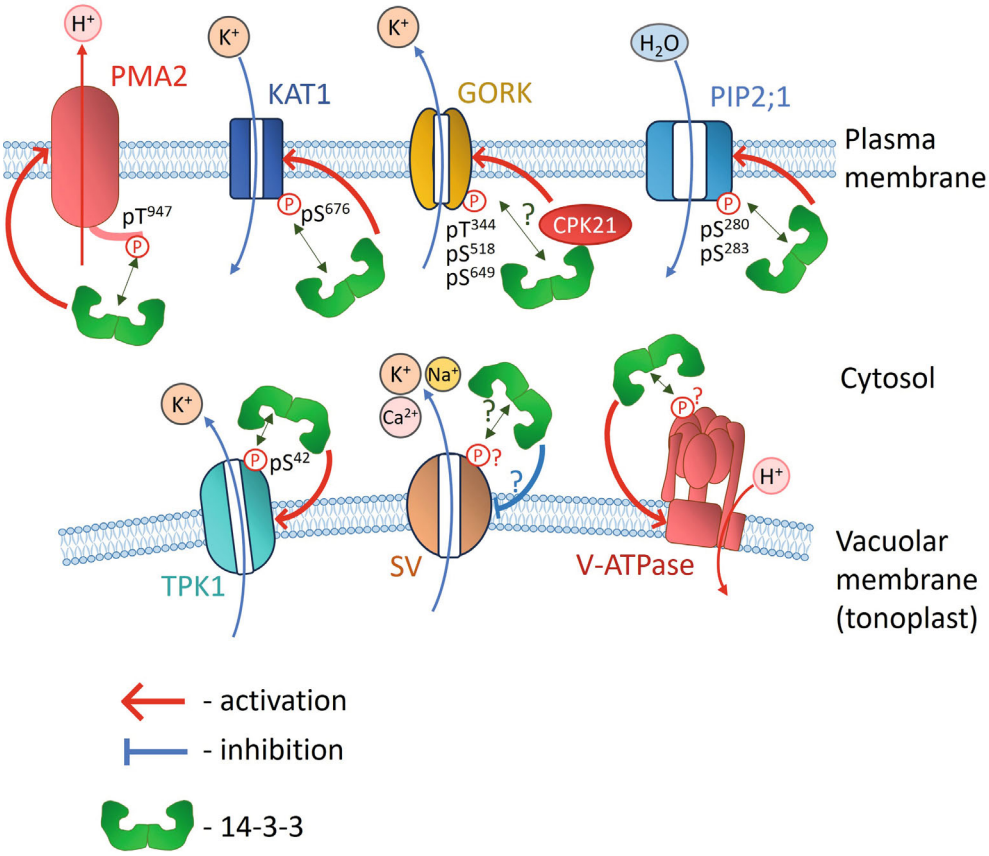

Fig. 8. The effect of 14-3-3 on the functions of membrane proteins. The binding of 14-3-3 to the cytosolic parts of plasma membrane integral proteins regulates their activity: the binding of 14-3-3 to PMA2 leads to the enzyme activation; the interaction with potassium channels KAT1 and GORK promotes ion currents through the channels; the binding to the aquaporin PIP2;1 maintains its open state. In the vacuole membrane, 14-3-3 interaction with the potassium channel TPK1 decreases the ion current through the channel; the binding of 14-3-3 to SV channels also slows down the cation current through them; the putative interaction of 14-3-3 with V-ATPase leads to the enzyme activation.

Another function of 14-3-3 proteins is the regulation of potassium ion transport across the plasma membrane. It has been shown that 14-3-3 enhances ion currents through the voltage-dependent potassium channel KAT1 (K^+ channel in *A. thaliana*) that conducts potassium into the cell [167, 168] (Fig. 8). Saponaro et al. [165] showed that 14-3-3 interacts with KAT1 through the C-terminal type III motif YFSpSN-COOH (phosphorylated residue in *A. thaliana* is Ser676) [165]. The authors obtained the spatial structures of *N. tabacum* 14-3-3 complex with the phosphopeptide from KAT1 (type III motif) in the presence and absence of fusicoccin [165]. The data suggested that 14-3-3 influences the opening of stomata via two parallel mechanisms: by acting via PMA ATPase to generate the proton gradient and hyperpolarize the membrane in order to promote ion current into the cells and by affecting KAT1 channels to directly increase K^+ entry into the cells [165]. Both processes ultimately lead to the entry of water into stomatal guard cells and their opening. The role of 14-3-3 proteins in the regulation of the functioning of stomatal guard cells is discussed in detail in the review by Cotelle et al. [147].

Van Kleeff et al. investigated interactions between 14-3-3 and GORK (Guard Cell Outward Rectifier K^+) channels from *A. thaliana* [169] (Fig. 8). These channels remove potassium ions from cells, thus participating in the stomata closure and maintenance of K^+ homeostasis in root cells [170]. Using pull-down assay, the authors showed that GORK channels interact with 14-3-3 λ, ν, and χ isoforms. However, they failed to confirm a direct binding of 14-3-3 with GORK channels in the yeast two-hybrid assay. It was found that 14-3-3 interacts with the calcium-dependent kinase CPK21 and activates its, while CPK21, in turn, phosphorylates GORK channels at Thr344, Ser518, and Ser649 [169]. The studies of mutant plants deficient by CPK21, 14-3-3 χ, and 14-3-3 φ have shown that these proteins are required to maintain normal potassium ion currents through the GORK channels [169].

14-3-3 proteins interact with aquaporins and regulate their functions. It was found that plant aquaporins from the PIP (Plasma membrane Intrinsic Protein)

group can bind 14-3-3 proteins [171, 172]. Aquaporins facilitate water transport across the membrane. Thus, PIP2;1 from *A. thaliana* regulates circadian fluid fluxes in leaves [173]. Using 14-3-3 knockout mutants, it was demonstrated that 14-3-3 is necessary for maintaining normal functions of PIP2;1 [172]. It is possible that PIP2;1 interacts with 14-3-3 through the Ser280 and Ser283 residues located closer to the C-terminus [172]. It is believed that phosphorylation of these sites has a regulatory function [173]. These studies again lack structural information that could shed light on the mechanism of aquaporin regulation by phosphorylation and 14-3-3 protein binding.

Regulation of transport across the vacuolar membrane. 14-3-3 proteins also regulate transport through the vacuolar membrane (tonoplast). In particular, 14-3-3 regulates potassium ion transport to the vacuole lumen through the channels of the TPK/KCO (tandem-pore K^+ channels/K_{ir}-like channels) family (Fig. 8). The functions of these channels in plants are poorly understood. They contain the 14-3-3 recognition motif I at the N-terminus at the cytosolic side of the membrane (phosphorylated residue in *A. thaliana* TPK1 is Ser42) and the EF-hand domain at the C-terminus [174]. The interaction between 14-3-3 λ and *A. thaliana* TPK1 was shown using the pull-down assay and the surface plasmon resonance method [175]. The binding of three 14-3-3 isoforms (14-3-3 A, B, and C) from barley (*Hordeum vulgare*) and HvKCO1 channel (a homolog of *A. thaliana* TPK1) was demonstrated using surface plasmon resonance [176]. The binding of 14-3-3 to TPK1 activates this channel [175]. Interesting data were obtained when studying TPK1 from *H. vulgare*. Two of the three isoforms, 14-3-3 B and C, inhibited this channel, while 14-3-3 A had no effect on the ion current [176]. Latz et al. showed that the serine residue in the 14-3-3 recognition motif in *A. thaliana* TPK1 is phosphorylated by the calcium-dependent protein kinase CPK3 [177]. The activity TPK1 is important for maintaining ion homeostasis during plant response to salt stress [177].

Slow cation (SV) channels are also regulated by 14-3-3 proteins (Fig. 8). These channels conduct Ca^{2+}, K^+, and Na^+ cations when activated by the increased intracellular Ca^{2+} concentrations [170]. 14-3-3 proteins slow the current through these channels [175, 178], although the details of interaction between 14-3-3 and SV channels remain unknown.

Another protein interacting with 14-3-3 is V-ATPase [179] (Fig. 8). V-ATPase performs a number of important functions in the vacuole membrane. By transferring protons across the membrane, it forms the electrochemical potential, controls turgor pressure, and stabilizes pH of the cytoplasm [180, 181]. Presumably, 14-3-3 interacts with the A subunit of V-ATPase [179], but the exact recognition site of 14-3-3 remains unknown. The amount of 14-3-3 proteins associated with V-ATPase increases upon illumination with blue light, as does the V-ATPase activity, but the direct effect of 14-3-3 on the enzyme activity has not been characterized [179].

From the above examples, it is evident that plant 14-3-3 proteins regulated the functions of many membrane proteins, including proton-transporting ATPases, ion channels, and aquaporins. Many of these 14-3-3 partner proteins are involved in controlling the activity of stomatal guard cells. Therefore, the opening/closing of stomata is regulated by 14-3-3 proteins not only during receiving and transmitting blue light signal via phototropins, but also directly through the regulation of ion currents.

14-3-3 proteins as regulators of signaling cascades. Having a sedentary lifestyle, plants cannot escape from stress factors and have to adjust biochemical characteristics of their cell to environmental conditions. The effects of various external factors have led to the formation of a branched signaling network designed to fine-tune the process of biochemical adaptation. By binding phosphorylated residues in proteins, 14-3-3 proteins can participate in protein kinase/protein phosphatase signaling cascades or to add another level of regulation.

Signaling pathways involved in biotic stress response. The mitogen-activated protein kinase (MAP) cascade is one of the best studied in plants. It is a linear cascade of sequentially phosphorylating kinases: MAP kinase kinase kinase (MAPKKK) phosphorylates MAP kinase kinase (MAPKK), which phosphorylates terminal MAP kinase (MAPK) and activates it [184]. The activation of this signaling pathway can lead to cell division and differentiation, cell death, and response to abiotic and biotic stresses [184]. Oh et al. [185] found that MAPKKKα from tomato (*S. lycopersicum*) interacts with the 14-3-3 isoform TFT7 in the phosphopeptide-binding groove of 14-3-3 [185]. A possible phosphorylated residue in MAPKKKα is Ser535, whose amino acid environment corresponds to the 14-3-3 recognition motif II [185]. The presence of 14-3-3 promotes the stability of MAPKKKα protein in cells and increases the amount of proteins phosphorylated by this kinase [185]. Overexpression of TFT7 promotes the pathogen-induced programmed cell death induced by MAPKKKα [185]. In another study, Oh et al. [75] showed that TFT7 binds *S. lycopersicum* MAPKK isoform SlMKK2 (MAPKKKα target) [75]. The putative recognition site for 14-3-3 is a sequence that includes phosphorylated Thr33 and corresponds to the type II motif [75]. In this case, however, the authors did not find a direct effect of TFT7 on the activity and stability of SlMKK2 [75]. It was suggested that 14-3-3 has an adaptor function, since its dimer contains two recognition sites for phosphorylated motifs, one of which

can bind SlMKK2, and the other binds MAPKKKα (as was shown previously), leading to the convergence of the two kinases and acceleration of signal transduction [75]. New data to the concept of MAP kinase cascade regulation by 14-3-3 were added by a recent study by Dong et al. [111], who demonstrated that 14-3-3 κ and λ interact with MAPKKK5 and PBL19 from *A. thaliana*. PBL19 is a protein kinase of the RLCK family that phosphorylates the C-terminus of MAPKKK5 and activates it, but the access of PBL19 to the phosphorylation site is limited by the autoinhibitory N-terminus of MAPKKK5. By binding to the C-terminus of MAPKKK5, 14-3-3 λ eliminates this inhibition and allows PBL19 to phosphorylate and activate MAPKKK5 [111] (Fig. 3e). Hence, 14-3-3 λ functions as a scaffold that facilitates MAPKKK5 activation [111], leading to the initiation of plant immune response against pathogens [111]. However, as in many other cases, there is no complete understanding of the MAPK cascade regulation by 14-3-3 proteins due to the lack of structural information on the relevant complexes.

The 14-3-3 involvement in the regulation of MAPK cascade explains an important role of 14-3-3 proteins in the plant immune response to pathogens. It was found that some effector proteins of pathogenic prokaryotes, eukaryotes and viruses can directly interact with 14-3-3 proteins of host plants. Thus, the coat protein (CP) of the beet black scorch virus (BBSV, a (+)RNA virus) interacts with 14-3-3a of *Nicotiana benthamiana* [186]. It was proposed that the recruitment of 14-3-3 by CP leads to a decrease in the activity of another 14-3-3 partner protein, MAPKKKα, which prevents initiation of cell death and promotes virus replication [186]. A similar effect was shown for the 14-3-3 partner protein XopQ from the phytopathogenic bacterium *Xanthomonas euvesicatoria*. Recruitment of 14-3-3 by XopQ resulted in the suppression of the MAPKKKα-mediated cell death, presumably due to the disruption of 14-3-3 interactions with proteins, including those with MAPKKKα [187]. Bacteria also secrete other 14-3-3-interacting effector proteins. For example, *Xanthomonas* bacteria produce XopX [188] and XopN1 [189], while *Pseudomonas syringae* secretes HopM1 [190] and HopQ1 [191]. The binding of 14-3-3 with these effector proteins suppresses plant immune response to pathogens and facilitates infection. The pathogenic oomycete *Phytophthora palmivora*, which causes rot diseases of many tropical crops, secretes the FIRE protein into the plant cell. FIRE interacts with 14-3-3 proteins of the host plant via the type I recognition motif, leading to successful infection; however, the molecular mechanism of this process remains unclear [192]. As can be assumed from the presented data, recruitment of cellular 14-3-3 proteins by binding to the effector protein is widely used by plant pathogens to suppress plant immune response, which characterizes 14-3-3 as an important element in the regulation of plant immunity [193]. However, both the details of such interactions and molecular mechanisms of 14-3-3 functioning in plant immune response have not been clarified so far.

Signaling pathways involved in response to abiotic stress. 14-3-3 proteins participate in the SOS (Salt Overly Sensitive) signaling cascade, which regulates plant response to salt stress. The main participants of the SOS cascade are the serine/threonine protein kinases SOS2 and PKS5 (SOS2-Like Protein Kinase 5), Na^+/H^+ exchanger of the plasma membrane SOS1, and calcium-binding protein SOS3 [194]. Under normal conditions, the activities of SOS2 and SOS1 are low. In the case of salt stress, the concentration of Ca^{2+} in cells increases, which induces SOS3 interaction with SOS2, SOS2 activation, and phosphorylation of SOS1. Activated SOS1 pumps sodium ions out of the cell, thus controlling salt homeostasis [195]. The role of 14-3-3 proteins in the SOS cascade was demonstrated by Yang et al. [62], who showed that under normal conditions, PKS5 phosphorylates SOS2 at Ser294, leading to its interaction with 14-3-3 λ and decrease in the SOS2 activity [62]. However, the amino acid environment of Ser294 ($DGIEGS_{294}YVAENV$) does not correspond to the canonical 14-3-3 recognition motifs. Using thermophoresis, the authors showed that 14-3-3 κ and λ are able to bind Ca^{2+} [62]. When the concentration of Ca^{2+} increases during the salt stress, 14-3-3 dissociates from SOS2, resulting in the increase in its kinase activity, SOS1 activation, and pumping of sodium ions out of cells [62]. Interestingly, the authors also showed that 14-3-3 λ interacts with PKS5 and inhibits it [62].

Phototropin signaling pathways. Another important role of 14-3-3 proteins in plants is mediated by their interaction with the phototropins PHOT1 and PHOT2. Phototropins are membrane-associated blue-light receptor protein kinases that initiate intracellular signaling cascade regulating the opening of the stomata, phototropism, and chloroplast movements [196, 197]. Phototropins consist of two LOV domains located at the N-terminus and responsible for capturing light quanta, and the C-terminal kinase domain that ensures signal transmission [196, 198]. During photoreceptor activation, a quantum of blue light is directly captured by flavin mononucleotide (FMN; absorption maximum, 447 nm) within the LOV domain. FMN covalently attaches to a cysteine residue, causing conformational changes leading to phototropin autophosphorylation and initiation of the signaling cascade [196, 198]. Sullivan et al. [199] analyzed interaction of PHOT1 and PHOT2 from *A. thaliana* with six 14-3-3 isoforms, four of which belonged to the non-epsilon group (κ, λ, υ, φ), and two were from the epsilon group (ε, ο). Using far-western blotting, it was demonstrated that PHOT1 specifically bound the non-epsilon

isoforms κ, λ, and φ, while the epsilon isoforms (ε, ο) did not interact with the protein on the membrane [199]. The authors showed that residues responsible for the interaction with 14-3-3 (Ser350, Ser376, and Ser410) were located between the two LOV domains [199]. At the same time, PHOT2 did not interact with 14-3-3 λ and κ [199]. In a later study, Tseng et al. used far-western blotting and yeast two-hybrid assay to demonstrate that PHOT2 interacts with 14-3-3 λ [200]. According to the yeast two-hybrid assay and functional studies in mutant plants, the residue responsible for the interaction with 14-3-3 was Ser747 located in the kinase domain [200]. The authors demonstrated that in *A. thaliana* plants, the effect of PHOT2 on the stomata opening was impaired by mutations in 14-3-3 λ, but not in 14-3-3 κ [200], indicating a narrow isoform specificity of 14-3-3 effects. In the absence of stringent controls, this causes some surprise, since the λ and κ isoforms are phylogenetically close and have 93% amino acid sequence identity [200]. The results of studies by Sullivan et al. [199] and Tseng et al. [200] on the interaction of 14-3-3 and PHOT2 contradict each other and suggest different 14-3-3 binding sites for such similar proteins as PHOT1 and PHOT2. A detailed study of the interaction of phototropins with 14-3-3 proteins using structural biology methods could clarify these issues and explain conflicting observations.

Phytohormone-induced signaling cascades. The signaling cascade of the gaseous phytohormone ethylene is also regulated by 14-3-3 proteins. In *A. thaliana* plants, 14-3-3 proteins interact with ACS, the key enzyme of ethylene biosynthesis. Yoon et al. [113] demonstrated that ACS coimmunoprecipitated with 14-3-3 ω, as well as confirmed this interaction *in vivo* for four tested 14-3-3 isoforms (ι, ο, κ, φ) using bimolecular fluorescence complementation method. Overexpression of 14-3-3 ω in cells increased the stability of ACS, while the treatment of plants with the R18 peptide (which inhibits the binding of 14-3-3 to other peptides by blocking the amphipathic groove) accelerated a decrease in the total ACS content, likely due to protein degradation. The authors also showed that 14-3-3 ω interacts with ETO1-like (EOL) proteins, components of the E3 ubiquitin ligase, which target some ACS isoforms for degradation [113, 201]. In a later study, Catalá et al. [202] also demonstrated the binding of 14-3-3 ψ (RARE COLD INDUCIBLE 1A, RCI1A) with ACS using coimmunoprecipitation and bimolecular fluorescence complementation assays [202]. However, the stability of the ACS protein in the presence of 14-3-3 ψ in cells did not increase (as in the case of 14-3-3 ω), but rather decreased. The authors explained this discrepancy by the specificity of the 14-3-3 ω and 14-3-3 ψ interactions with their partner proteins [202]. In our opinion, these undoubtedly interesting data require systematization and new studies, in particular, with the use of structural biology methods, in order to explain the observed discrepancies.

Plant 14-3-3 proteins as regulators of gene expression. One of the first names of plant 14-3-3 protein – G-box-associated factor (GF14) – was due to the discovery of this protein in a complex with the G-box element in DNA [7], which suggests participation of 14-3-3 proteins in the regulation of gene transcription.

In *A. thaliana*, 14-3-3 was found to regulate transcription factors BZR1 and BES1, which are components of the brassinosteroid (plant hormone) signaling pathway. In the absence of brassinosteroids, BZR1 and BES1 are phosphorylated by the GSK3 family protein kinase BIN2 at Ser173 [102] and Ser171 [101], respectively. The amino acid environments of the phosphorylated residues represent type II motifs. Phosphorylation results in the binding of 14-3-3 (λ and ω isoforms for BZR1 [26, 102] and λ isoform for BES1 [101]), resulting in the retention of the partner proteins in the cytoplasm and prevention of their entry to the nucleus (Fig. 3c). In the presence of brassinosteroids, the signaling cascade from the receptor complex of BRI1 (Brassinosteroid Insensitive 1) and BAK1 (BRI1-Associated Kinase 1) leads to the inhibition of protein kinase BIN2 by the BSU1 phosphatase [203]. This causes dephosphorylation of BZR1 and BES1, dissociation of their complexes with 14-3-3, and import of BZR1 and BES1 into the nucleus, where they initiate transcriptional programs required for the response to brassinosteroids [101, 102]. Hence, 14-3-3 is involved in the cytoplasmic sequestration of transcription factors, possibly due to the presence of the nuclear export sequence (NES) in the C-terminal region of 14-3-3, as has been shown for human and yeast 14-3-3 partner proteins [50, 52].

14-3-3 proteins can be directly involved in the assembly of transcriptional complexes. It was shown in *O. sativa* suspension culture and *Zea mays* embryos, that 14-3-3 is a component of the protein complex that binds the DNA sequence of the *Em1a* regulatory element in the *Em* gene promoter, whose transcription is activated by signaling initiated by the phytohormone abscisic acid [110]. The complex also includes transcription regulators Viviparous1 (VP1) and EmBP1 (b-ZIP family factor) [110]. The association of 14-3-3 with the *AtEm1* gene promoter was also shown in *A. thaliana* embryonic cell culture [204].

An important role of 14-3-3 proteins is regulation of flowering at the stage of florigen-activating protein complex formation. The GF14c isoform from *O. sativa* interacts *in vitro* and *in vivo* with the so-called florigen, or Hd3a (heading date 3a) protein, whose homolog in *A. thaliana* is encoded by the *FT* (FLOWERING LOCUS T) gene [41, 96]. Florigen is synthesized in the leaves and transported to the apical meristem of shoots, where it enters the cell cytoplasm

and interacts with GF14c. The resulting complex enters the nucleus and binds to the transcription factor OsFD1 (FD is OsFD1 homolog in *A. thaliana*) [96]. The florigen-activating complex (FAC) formed by the GF14c dimer, OsFD1 dimer, and two Hd3a proteins, activates transcription of OsMADS15 (its homolog in *A. thaliana* is called *APETALA1*, or *AP1*), thus triggering the flowering [96]. The spatial structure of a FAC fragment was solved by X-ray crystallography; it is the only structure of plant 14-3-3 complex with a full-length partner protein in the PDB (PDB ID: 3AXY; 164 amino acid residues out of 179 resolved in Hd3a structure) (Fig. 7b) [96]. OsFD1 is phosphorylated at Ser192 (type I motif) and bound in the groove of 14-3-3 (as follows from the structure of 14-3-3 complex with a OsFD1 phosphopeptide) [96]. In *A. thaliana*, FD transcription factor is phosphorylated at Thr282 (residue homologous to Ser192 in OsFD1) by calcium-dependent protein kinases, such as CPK6 and CPK33 [205].

14-3-3 proteins can indirectly regulate gene expression by affecting the stability of transcription factors. This regulatory mechanism has been shown for the transcription factor WRINKLED1 (WRI1), a master regulator of triacylglyceride biosynthesis [114]. WRI1 likely interacts with 14-3-3 at the site that was also predicted to bind E3 ubiquitin ligase [114]. Hence, 14-3-3 can protect WRI1 from proteasomal degradation and promote expression of WRI1-regulated genes of triglyceride biosynthesis [114] (Fig. 3f).

Guo et al. showed that 14-3-3 (HbGF14a isoform) from the rubber tree (*Hevea brasiliensis*) regulates the activity of the transcription factor HbRZFP1 (*H. brasiliensis* RING zinc finger protein) [206]. HbRZFP1 binds to the promoter of the gene coding for Hevea rubber transferase (HRT2), an enzyme that adds isoprene units to the growing rubber polymer, and inhibits its expression. HbGF14a has been shown to interact with HbRZFP1 *in vivo* and *in vitro* in bimolecular fluorescence complementation, yeast two-hybrid, and pull-down assays [206]. A regulatory mechanism has been proposed in which the binding of HbGF14a to the transcription factor HbRZFP1 disrupts HbRZFP1 interaction with the HRT2 gene promoter, resulting in upregulated expression of this transferase [206]. Several studies have also investigated the role of 14-3-3 proteins in rubber biosynthesis. HbGF14c (another 14-3-3 isoform from *H. brasiliensis*) has been shown to interact with the small rubber particle protein (SRPP), which is present in rubber particles and involved in rubber biosynthesis [207, 208]. However, the details of this interaction and the corresponding regulatory mechanism remain unknown.

As can be seen from the above, 14-3-3 proteins participate in the regulation of many transcription factors and DNA–protein complexes. However, the effects and the mechanisms of this regulation are often poorly understood, while the details of protein–protein interactions still remain enigmatic. Using modern methods of structural biology and studying the processes at the protein level might help in resolving these issues.

CONCLUSION. "BLANK SPOTS" IN THE STUDIES OF PLANT 14-3-3 PROTEINS

As can be seen from the data presented in this review, plant 14-3-3 proteins are involved in the regulation of many important biochemical and physiological processes. The fact of 14-3-3 family involvement in these processes is beyond any doubt. Hundreds of research papers and dozens of reviews have been devoted to the functions of 14-3-3 proteins in plants. However, despite more than thirty years of research, many issues about the structure and functions of plant 14-3-3 proteins remain unexplored. The majority of these fundamental problems arise due to the lack of systematic approach and, as a consequence, a fragmentary nature of the obtained data. 14-3-3 proteins are a multigene family, and plant species often have several 14-3-3 isoforms, so to obtain a complete picture, it is necessary to study either all representatives of the family or a representative set of these isoforms. In the first case, the task is extremely labor-intensive even for plant species with a relatively small number of isoforms. In the second case, it is unclear which criteria should be used to select 14-3-3 isoforms for analysis. Therefore, in most studies, isoforms are chosen at random, which complicates data interpretation and makes global generalizations impossible. Below, we present some of the most interesting unresolved issues – "blank spots" – in the field of plant 14-3-3 research.

Are biochemical properties of plant 14-3-3 homodimers similar or different? One of the fundamental questions is related to the biochemical characteristics of 14-3-3 proteins. These proteins are quite conserved; the pairwise identity of amino acid sequences of 14-3-3 isoforms from *A. thaliana* is 60% on average and reaches 80-90% in some cases. However, the question whether their biochemical characteristics are the same has not been properly addressed. When we started to investigate this problem, we were surprised to find that 14-3-3 isoforms from the epsilon and non-epsilon subgroups in *A. thaliana* differ significantly in the stability of formed homodimers, hydrophobicity of protein surface, thermal and proteolytic stability, and other properties [209]. Thus, unlike non-epsilon isoforms, isoforms from the epsilon group are prone to dissociation into monomers, have a lower half-transition temperature (the greatest difference in the half-transition temperature between the epsilon

and non-epsilon isoforms was over 20°C), and are more susceptible to limited proteolysis [209]. Even the homodimers of plant 14-3-3 proteins can significantly differ from each other. The search for such distinctive features and biochemical characteristics is of fundamental importance, since they can strongly impact the half-lifetime (stability) and biological functions of 14-3-3 isoforms in plants. Also, understanding specific properties of isoforms from different subgroups can help in phylogenetic analysis and projecting the properties inherent in a subgroup or in individual isoforms in some plants onto other less-studied isoforms (and in less-studied plants). In the absence of such information, the growing body of data cannot be properly systematized and understood.

Which plant 14-3-3 isoforms form heterodimers? Is there a preference for particular isoforms during heterodimer formation? Heterodimerization of plant 14-3-3 proteins, both *in vitro* and *in vivo*, has been demonstrated in several studies. It was found that 13 isoforms of *A. thaliana* 14-3-3 proteins can theoretically form 78 different heterodimers, but only 11 of them have been reported to generate heterodimers, all of these isoforms belonging to the non-epsilon phylogenetic group. Moreover, there is a lack of information on the ability of plant 14-3-3 proteins to heterodimerize in their native form, without the use of denaturation/renaturation. It remains unknown whether plant 14-3-3 isoforms from the epsilon subgroup heterodimerize with each other, and whether epsilon and non-epsilon isoforms can form heterodimers. The biological function of heterodimerization and advantages it provides (if any) remain obscure.

Is there a hierarchy of affinity of plant 14-3-3 proteins for phosphorylated peptides? What is the reason for the differences in the affinity of 14-3-3 isoforms? It has been shown that regardless of the type of the partner protein, the hierarchy in the affinity of animal 14-3-3 isoforms remains more or less the same [92]. It is unknown whether this is true for plant 14-3-3 proteins. Several studies have shown that 14-3-3 isoforms from *A. thaliana* bind phosphopeptides (or partner proteins) with different affinity. A systematic approach is needed to figure out the patterns in the affinity of plant 14-3-3 proteins, since none of the studies have examined the full set of isoforms even for such model plant as *A. thaliana*. The maximum number of isoforms analyzed in one study was 9 (out of 13). The reason for the difference in the affinity between the 14-3-3 proteins still has to be elucidated. The residues exposed in the ligand-binding groove of 14-3-3 and responsible for the binding of phosphopeptides are fully conserved, so it remains obscure what provides the differences in the binding affinity from the structural point of view. This question is relevant for both plant and mammalian 14-3-3 proteins.

How widespread and universal is the regulation of plant 14-3-3 proteins by phosphorylation? What are the functional effects of phosphorylation? 14-3-3 proteins can undergo phosphorylation, which regulates their binding to the partner proteins and the 14-3-3 dimer structure [74, 82]. Large-scale phosphoproteomic studies have identified dozens of residues capable of being phosphorylated in plant 14-3-3 proteins [83, 210]. However, only two sites located at the 14-3-3 dimer interface have been functionally characterized. It also remains unclear to what extent the regulation by phosphorylation is conserved and universal across plant 14-3-3 isoforms.

Are the functions of plant 14-3-3 isoforms specific or redundant? This question has already been raised in several reviews on plant 14-3-3 proteins [46, 211, 212]. Does each 14-3-3 isoform bind specific partner proteins or can all 14-3-3 isoforms interact with all formally suitable partner proteins and compensate for each other's functions? A clear answer to this question is currently lacking. For example, 14-3-3 κ and λ isoforms from *A. thaliana* have appeared as a result of a relatively recent duplication of a single gene [14]. Their amino acid sequences are as high as 93% identical, which is the highest identity of the primary structures of 14-3-3 isoforms in *A. thaliana*. For such closely related isoforms, one would expect a strong overlap of functions and the least degree of subfunctionalization. This is consistent with some experimental data characterizing the functions of these isoforms as redundant. Thus, single mutations in either 14-3-3 λ and κ had no effect on the flagellin-induced expression of marker genes [111]. Both single mutants, as well as the double mutants by 14-3-3 λ and κ, did not differ from the wild-type plants in the root length [36]. However, other studies have demonstrated distinct properties and effects of these highly related isoforms: 14-3-3 λ (but not κ) bound RPW8.2 protein in immunoprecipitation experiments [213]; 14-3-3 λ (but not κ) was shown to play a role in the PHOT2-mediated opening of stomata *in vivo* [200]; both 14-3-3 λ and κ interacted with the PHOT1, but 14-3-3 λ bound the protein several-fold more strongly than 14-3-3 κ [199]; the affinity of 14-3-3 λ for various targets was consistently higher than that of 14-3-3 κ [58]. Thus, even very similar 14-3-3 isoforms can have both specific and overlapping functions. Apparently, there is no general rule about redundancy or specificity of plant 14-3-3 proteins, and the extent of manifestation of the functional effects of particular isoforms depends on specific protein–protein interactions.

Lack of information on plant 14-3-3 partner proteins. Another serious problem is the lack of information on the interactions of 14-3-3 isoforms with partner proteins, in particular, on the 14-3-3 binding site, functional consequences of such interactions,

and spatial structures of complexes formed. Solving a spatial structure naturally helps to identify the mechanism of regulation of partner proteins by 14-3-3 isoforms. At present, only two structures of plant 14-3-3 complexes with a partner protein (florigen Hd3a from *O. sativa*) or a large fragment (C-terminal fragment of the plasma membrane H^+-ATPase PMA2 from *N. plumbaginifolia*) have been determined [96, 99] (Fig. 7, a and b). The details of how 14-3-3 proteins affect their interaction partners are either unknown or incomplete, which leads to ambiguity in the interpretation of experimental data and contradictions. For example, neither the phosphorylated residue (Ser229) in SPS from *S. oleracea* originally identified by Toroser et al. [98], nor the inhibitory effect of 14-3-3 upon binding to the enzyme have been confirmed in later studies. The regulatory mechanism has been most fully described for the two classical 14-3-3 partner proteins – NR [124] and PMA [99], but for many other proteins, the details of interaction with 14-3-3 remain unknown. Thus, there are no data on the 14-3-3 binding sites in GAPN, ACS, GS1, and transcription factors WRI1, VP1, and EmBP1, as well as on the kinases that phosphorylate them. Based on the general concepts of 14-3-3 interaction with partner proteins, it might be possible to fully describe the regulatory mechanism if the following information is revealed: (i) 14-3-3 recognition site in the partner protein; (ii) kinase that phosphorylates it; (iii) effect of 14-3-3 binding on the activity and conformation of the partner protein *in vitro*; (iv) functional consequences of this interaction *in vivo*. Only by answering all these questions, researchers will be able to resolve existing contradictions and clarify the mechanisms of 14-3-3-regulated processes in plants.

Is yeast two-hybrid assay applicable to studying 14-3-3 interactions with partner proteins? Yeast two-hybrid assay has been repeatedly used in the studies of 14-3-3 proteins. The screening of cDNA libraries has revealed tens and hundreds of protein–protein interactions [171, 214]. However, the method may not be suitable for testing specific binary interactions due to specific features of 14-3-3 interaction with partner proteins. The binding of 14-3-3 requires phosphorylation of the partner protein at a specific motif by a specific kinase, which might exist in plants only. The formed protein complexes might not localize to the nucleus, for example, if the partner protein is located in the membrane or near it. These features complicate the use of yeast two-hybrid assay and limit its applications in the studies of protein–protein interactions of 14-3-3 isoforms. For example, the conclusion about the absence of direct binding between 14-3-3 and GORK channels based on the negative results of yeast two-hybrid assay is questionable, as in the same study, specific binding of these two proteins was demonstrated by the pull-down assay [169]. Therefore, yeast two-hybrid assay should be used with great caution and, preferably, in combination with other methods when studying protein interactions involving 14-3-3 proteins.

Can plant 14-3-3 proteins regulate the functioning of partner proteins through the chaperone-like activity or by influencing the LLPS? A number of mechanisms by which the binding to 14-3-3 changes the biological function of partner proteins have been described for both plant and mammalian 14-3-3 proteins (Fig. 3, a-e). However, the chaperone-like activity and effect on LLPS have been found only for mammalian 14-3-3 proteins [117-119] (Fig. 4, b and c). Since plant 14-3-3 proteins are structurally similar to their mammalian homologs and have the same biological significance, it is reasonable to assume that similar mechanisms can be found in plants as well. In plants, LLPS has been described for several important physiological processes, including formation of molecular condensates in the nucleus during regulation of flowering and initiation of transcriptional response in the phytochrome phyB signaling [215]. 14-3-3 proteins are directly involved in the latter two processes and may play a role in the occurring LLPS events.

Are 14-3-3 proteins present in chloroplasts and mitochondria and how do they get there? 14-3-3 proteins are typically located in the cytosol and nucleus and lack a signal sequence for their import into chloroplasts [216]. However, several early studies have shown that plant 14-3-3 proteins can localize to the chloroplast stroma [44, 106]. Moreover, it has been shown that 14-3-3 isoforms can interact with and regulate the activity of several chloroplast proteins, such as GS2 [129], starch synthase [44], and chloroplast and mitochondrial ATP synthases [217]. It remains unknown how 14-3-3 proteins get into the double-membrane organelles and what functions they perform there. These issues, addressed mainly in early works, need to be reconsidered and investigated using modern methods of biochemical and structural analysis.

The problems of phenotypic analysis of plants mutant for 14-3-3 proteins. Phenotypic analysis of mutants could provide opportunities for identification and characterization of specific functions of 14-3-3 isoforms in plants. The knockouts, overexpression, and heterologous expression of 14-3-3 proteins in different plant species have been extensively investigated [18, 25, 37-45, 169], however, the studies on 14-3-3 mutants are often unsystematic and incomplete. The lack of systematic approach is evidenced by the fact that there are no published studies where single mutants for all 13 isoforms in *A. thaliana* have been phenotypically characterized. The isoforms for studying are often chosen randomly; an isoform of one plant can be expressed in another plant. The studies are often focused on a particular trait of mutant plants

and ignore the others (there are very few or no studies of the "one isoform-many traits" or "one trait-all isoforms" type). As noted above, the main phenotypic manifestations of 14-3-3 mutations are changes in plant growth, timing of transition to flowering, tolerance to salt stress, and starch accumulation, i.e., traits important in agriculture. We assume that the authors of such studies reported only prominent and omitted less obvious effects of mutations. Interpretation of results of knockout studies can be significantly complicated by the compensatory effect of other 14-3-3 isoforms, which certainly occurs in the case of fairly similar 14-3-3 proteins. The scope and fundamental significance of studies on the reverse genetics of plant 14-3-3 proteins could be greatly increased by using a systematic approach and comprehensive analysis of 14-3-3 mutants.

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