Absciscic acid (ABA) signaling: Finding novel components off the beaten track

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Abstract

The sesquiterpene abscisic acid (ABA) is an ancient universal stress response molecule. In plants, many ABA-dependent processes operate via the PYR/PYL/RCAR receptor complexes, however, there is evidence suggesting that not all plant responses operate through this mechanism. This is consistent with ABA responses in animals and humans which do not contain such receptors. We hypothesize that plant and animal proteomes harbour proteins with undiscovered ABA-binding sites and propose that carefully curated amino acid search motifs deduced from the binding sites of experimentally confirmed ABA-binding proteins can identify many more candidates in plant and animal proteomes many of which show folds that are compatible with ABA-binding. Plant candidates include annotated ABA downstream signaling components such as SnRK2.2 and SnRK2.6 and proteins with roles in protein folding and RNA polyadenylation, while candidates in the human proteome affect, among other processes, immune responses and tumor progression. If these candidates will eventually be experimentally validated, it will imply that the regulation and tuning of ABA-dependent processes is considerably more complex than hitherto suspected and it will also expand and help to clarify the role of ABA in animal systems.

Introduction

Since the discovery of the chemical nature of abscisic acid (ABA) (Cornforth et al. 1965a, b) and reports of ABA-dependent physiological responses in higher plants (Mittelheuser and van Stevening 1969), over half a century has passed and an ever growing body of scientific literature (>10,000 articles in PubMed) has reported stimulus-induced ABA synthesis, and ABA-dependent signaling and physiological responses at the molecular and systems level (for recent contributions see e.g., (Nakashima and Yamaguchi-Shinozaki 2013; Wang et al. 2015; Chong et al. 2019; Chen et al. 2020; Takahashi et al. 2020; Lin et al. 2021)) across the kingdoms of life (Takezawa et al. 2011; Lievens et al. 2017). We can be confident that the molecular resolution of spatial and temporal ABA-dependent processes known to-date will steadily increase, we can, however, be much less confident that there are no hidden mechanisms that may operate entirely without the currently known key signaling components, notably the PYL, PP2C-A, and SnRK2 (Ali et al. 2020; Lin et al. 2021).

Plant ABA synthesis and signaling in different mutualistic and pathogenic systems and the particular role of ABA as modulator of the host immune response has been reviewed extensively (Lievens et al. 2017). What remains intriguing is the fact that ABA is also present in animals (Le Page-Degivry et al. 1986) and given that animals do not have a PYR/PYL/RCAR signaling system, they may provide us with important clues for the elucidation of alternative ABA-dependent mechanisms and processes.

ABA has been reported in the early metazoans (Porifera) (Zocchi et al. 2001) and it was shown that firstly, heat causes ABA production, and secondly, ABA, via stimulating a protein kinase-A (PKA), induces ADP-ribosyl cyclase activation through phosphorylation, and finally, the resulting cADPR generation causes Ca^{2+} release. Another report demonstrates that ABA, naturally taken up via the nectar (Lipp 1990), has a number of beneficial effects in bees (*A. mellifera*) including the boosting of their innate immune defence

and possibly increased tolerance to pesticides (Negri et al. 2015). ABA also enhances cold tolerance in honeybee larvae concomitant with modulated expression of genes encoding stress response proteins, and proteins diagnostic for metabolic adjustments.

ABA effects have also been investigated in human granulocytes, insulin-producing rat insulinoma cells, and by human and murine pancreatic β cells which produce and release ABA and induce a signal cascade via components including G-protein complexes, cAMP, cADP-ribose and intracellular Ca^{2+} transients. Perhaps the most significant contribution to our understanding of ABA signaling in animal cells was the discovery of ABA binding to the lanthionine synthetase C-like protein 2 (LANCL2) and its role in granulocytes and rat insulinoma cells (Sturla et al. 2009). It may also not be a coincidence that the animal LANCL protein family shares structural similarities with the prokaryotic lanthionine synthetase component C proteins which are bacterial membrane associated proteins that operate in the synthesis of antimicrobial peptides (Bauer et al. 2000). It has also been suggested that since LANCL2 links ABA interactions to anti-inflammatory and anti-diabetic responses, it might support the development of synthetic ABA agonists (Sturla et al. 2009). ABA transport has also been studied in human erythrocytes and shown to occur via a Band 3 anion transporter and that once inside, ABA can stimulate ATP release through the LANCL2-mediated activation of an adenylate cyclase (Vigliarolo et al. 2015) and hence cAMP. ABA does also affect inflammatory processes and again LANCL2 is a molecular target for ABA with subsequent G-protein linked cAMP accumulation in immune cells (Bassaganya-Riera et al. 2011) and last but not least, ABA has also been linked to tumor dormancy and reactivation (Jung et al. 2021).

In Search Of Undiscovered ABA-binding Proteins

Here we propose that plant and animal proteomes contain proteins that harbour hitherto undiscovered ABA-binding sites and that binding of ABA may specifically modulate their properties (e.g., enzyme activities) thereby modulating biological processes essential for growth, development and adaptation. This proposition is essentially based on the observation that ABA can specifically bind to components other than the PYR/PYL/RCAR complex and mediate ABA-specific effects. Three of many examples illustrate this point. Firstly, an affinity-based chemical proteomics approach has led to the identification of a mitochondrial adenine nucleotide translocators (ANTs) as ABA binding *in vitro* (Kharenko et al. 2011). Subsequent analyses demonstrated that ANT mediated ATP translocation was inhibited by ABA. The results are therefore entirely consistent with a direct role of ABA on mitochondrial ATP translocation across the matrix and a direct and rapid role of ABA in plant energy metabolism. In a later series of experiments an ABA mimetic probe combined with *in vitro* binding assays in *Arabidopsis thaliana* identified additional candidate ABA-binding proteins including Rubisco (Galka et al. 2015) directly linking ABA to CO_2 photosynthetic metabolism and hence affecting the pool size of photosynthetic intermediates and carbon metabolism in general (Suzuki et al. 2012). Secondly, a similar affinity-based method employing biotin linker containing both alkyne and amino groups, a protein cross-linker, and ABA azido probe, that circumvents the reduction in biological activities associated with direct attachment of large functional groups to ABA, has identified a thioredoxin from *Arabidopsis thaliana* (AtTrxh3), as an

ABA-binding protein. However, *in vitro* data showed no influence of ABA on the thioredoxin and chaperonin activities of AtTrxh3 and the AtTrxh3-mediated physiological effects of ABA-binding to AtTrxh3 remains to be elucidated (Anabuki et al. 2022). It is conceivable that binding of ABA to AtTrxh3 could affect biological processes such as seed germination, as well as plant adaptations to adaptations to stresses such as heat shock and cold (Lee et al. 2009; Li et al. 2009), which would be consistent with a regulatory or tuning role of ABA. Thirdly, ABA also binds directly and specifically to the *Arabidopsis thaliana* guard cell outward rectifying K⁺ channel GORK (At5g37500) and directly enhances K⁺ efflux (Ooi et al. 2017) and consequently promoting stomatal closure as it occurs e.g. at the onset of biotic or abiotic stresses. Incidentally, a stelar K⁺ outward rectifying channel (SKOR, At3g02850) also contains a GORK-like ABA-binding site and may also enable direct ABA binding causing K⁺ efflux.

What these and other examples point out is that firstly, direct ABA-binding is not likely an ancient relic, since it occurs e.g., in stomata and the vascular bundle and secondly, that the PYR/PYL/RCAR family and associated PP2C phosphatases are likely not the only mechanism through which specific ABA-dependent responses occur. Moreover, there is also considerable interest in ABA-binding in species other than plants and therefore reasons to extend the hypothesis to animal systems. The synthesis and signaling of ABA in different mutualistic and pathogenic systems and in particular the role of ABA as modulator of the host immune response, has been extensively reviewed (Lievens et al. 2017). What remains particularly intriguing is the fact that ABA is also present in animals (Le Page-Degivry et al. 1986) and given that animals do not have a PYR/PYL/RCAR signaling system, they may provide us with important clues for the elucidation of alternative ABA-dependent mechanisms and processes. Thus, a systematic approach beginning with sequence analyses of known ABA-binding proteins from both animals and plants would be a rational approach to capturing a more comprehensive ABA-binding protein repertoire (Wong et al. 2018).

How To Establish An ABA-binding Protein Repertoire

Firstly, we propose to employ a computational method to identify candidate ABA-binding proteins that is based on the use of consensus amino acid search terms carefully constructed from binding sites of annotated or experimentally validated ABA receptors or ABA-binding proteins. These kind of motif searches have in the past been applied to identify the first plant mononucleotide cyclases (Ludidi and Gehring 2003), and more recently novel plant phosphodiesterases (Kwiatkowski et al. 2021) as well as gas sensing hemoproteins (Wong et al. 2021).

Since a number of experimentally tested ABA-binding proteins and sites have been established, we can investigate them for conserved residues in their binding domain, extract a consensus search term and query proteomes for candidates. Such a conserved term [D.{7,8}R.{3,4}D.{5,6}Y.{6,7}H (Motif I)] that resembles the latch part of PYR/PYL/PCAR ABA receptors as well as the GORK ABA-binding site (Figure 1A), was extracted, and used to query the *Arabidopsis thaliana* proteome. It occurs in 44 proteins (see Supplementary Information). These 44 proteins are enriched in the gene ontology (GO) classes of

proteins with a role in “Protein folding”, “Heat shock protein binding” and “Unfolded protein binding” (Figure 1B and Supplementary Information).

If the central proline is omitted from the motif (Motif II), we found that the motif occurred in 331 proteins in the proteome (see Supplementary Information) and noted enrichments in the GO categories “RNA polyadenylation” and “Protein folding” as well as “Nitrogen compound metabolic process (Figure 1C). This may point to a direct role of ABA in these processes. In addition, we also noted ABA-binding sites *in bona fide* ABA downstream components and proteins associated with responses to ABA, and this might point to an increased complexity in the tuning of ABA-dependent responses (Figure 2) that awaits experimental testing *in vitro* and *in planta*.

Secondly, candidate ABA-binding proteins identified with the ABA search motif will have to be modelled and assessed structurally (Pettersen et al. 2004; Trott and Olson 2010; Jumper et al. 2021) to establish if a fold that is compatible with ABA-binding could be formed. A series of computational approaches that include molecular docking simulations can be performed to evaluate the ABA docking poses, the predicted free energies, and the interactions with key amino acids in the ABA motif. Both visual structural assessments and the statistical values generated by the docking algorithms, lend confidence to the selection of the most promising candidates for *in vitro* testing. For instance, structural evaluations of representative H-NOX candidates from *H. sapiens*: son of sevenless homolog 2, SOS2 (UniProt: Q07890), and exostosin-1, EXT1, and from *A. thaliana*: serine/threonine-protein kinase, SRK2D/SnRK2.2 (UniProt: Q39192), and serine/threonine-protein kinase, SRK2E/SnRK2.6 (UniProt: Q940H6), revealed that the predicted ABA-binding region identified by the motif, could form a clear cavity that can spatially fit the ABA ligand. Subsequent docking simulations on these candidates showed that ABA could dock at these sites and assuming binding poses that allow for interactions with at least two key amino acids in the motif (Figure 3). These key amino acids and several others in close proximity to the ABA ligand at the binding site, can be selected for mutagenesis studies to ascertain the molecular and biological relevance e.g., the intra- and inter-domain regulations of cellular signalling components, as well as the broader effects affecting plant development and responses to biotic and abiotic stresses (Zhou et al. 2021).

Thirdly, candidate ABA-binding proteins need to be cloned, heterogeneously expressed and tested *in vitro* for binding with both the physiologically active ($\pm$)ABA and inactive (-)ABA e.g., by surface plasmon resonance or ELISA. In candidates with multiple domains, the effects of ABA-binding on the neighbouring domains will also need to be investigated through e.g., mutagenesis studies. If a neighbouring domain has e.g., kinase or adenylate cyclase activity, the effect of ABA-binding on the enzyme activity will have to be tested in order to gain insight into specific ABA-dependent biological roles.

Conclusions

If many more direct ABA interactors exist and test positive in ABA-binding experiments, and shown to respond to ABA, this will imply that the regulation and tuning of ABA-dependent processes is considerably more complex than currently known. In plants, candidates that immediately spring to mind are

downstream signaling components of the canonical PYR/PYL/RCAR receptors such as SnRK2.2 and SnRK2.6. Establishing direct and specific ABA-binding to proteins with a role in RNA metabolic processes and RNA polyadenylation does point to a systems level role of ABA where modifications of the transcriptional program may turn out to be under direct ABA control. As such, the biological effects of experimentally validated ABA-binding proteins need to be investigated *in vivo* using transgenic cells or plants expressing proteins harboring mutations at the ABA-binding sites with reduced or abolished ABA binding.

In animals, predicted ABA-binding candidates include proteins with a role in immune responses and tumorigenesis (see Supplementary File). The establishment of an experimentally confirmed *H. sapiens* ABA-binding protein repertoire will expand and clarify the current role of ABA-dependent processes including immune responses and tumor cell progression. Finding hidden ABA-binding sites will in turn, guide further refinement of the ABA search motif, thereby facilitating the discovery of more ABA interactors and enable a broader understanding of this hormone in organisms across species.

Declarations

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Competing interests

The authors declare no competing interests.

Author contributions

C.G. conceived of the project, A.W. and C.B. performed the modeling and interpretation of the models. All authors contributed to the interpretation of the data and C.G., S.P. and A.W. wrote the manuscript.

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Figures

Figure 1

The ABA-binding motif and GO categories of candidate ABA-binding proteins. Alignment of canonical PYR/PYL/PCAR latch portion of ABA receptors of *Arabidopsis thaliana*, *Glycine soja* and *Medicago trunculata* and the ABA-binding GORK channel in *Arabidopsis thaliana*, *Cannabis sativa*, *Terenaya hassleriana* and *Capsella rubella*. The highlighted amino acids (blue) represent residues critical for interacting with ABA. The proline (D in green) is also conserved but does not appear to be critical for

interaction with ABA. Asterisks denote conserved amino acids. The two consensus motifs (I and II) used to query proteomes are shown below **(A)**. The gene ontology (GO) analyses was performed with agriGO (<http://bioinfo.cau.edu.cn/agriGO/>) and selected enrichments are shown in **(B and C)** and the extended result is shown in the Supplementary Information.

Figure 2

ABA-binding proteins and selected ABA-binding candidates. The experimentally proven ABA-binding proteins are bound to ABA marked “red”, candidate proteins are marked “magenta”. The candidates include downstream ABA signaling components PP2C (At3g17090) and SnRK2.2 (At3g50500) as well as SnRK2.6 (At4g33950, Open Stomata 1, OST1). Other ABA-binding candidate proteins include APRR2 (At4G18020), a pseudo response regulator that interacts with calmodulin-like proteins 8,9 &11 and the transcription factor TCP19 (At5g51910) that is annotated as regulator of defense responses. The binding candidate ACT-domain repeat ACR8 (At1g12420) implicated in abiotic stress responses and ATRSH2 (At3g14050) that is implicated in guanosine 3',5'-bis(pyrophosphate) (ppGpp) homeostasis.

Figure 3

Structural evaluation of selected candidate ABA-binding proteins from *H. sapiens* and *A. thaliana*.

Structures of two representative human H-NOX candidates: son of sevenless homolog 2, SOS2 (UniProt: Q07890) (**A**) and exostosin-1, EXT1 (UniProt: Q16394) (**B**), and two representative *Arabidopsis* H-NOX candidates: serine/threonine-protein kinase, SRK2D/SnRK2.2 (UniProt: Q39192) (**C**) and serine/threonine-protein kinase, SRK2E/SnRK2.6 (UniProt: Q940H6) (**D**), were represented as surface models with the region corresponding to the ABA-binding amino acid motif marked in pink, and the conserved amino acids colored according to their surface charges. In all candidates, the ABA-binding region forms a clear cavity that can be docked with the ABA ligand. The conserved amino acids in the motif that might interact with ABA, are labelled accordingly. For candidates where their crystal structures were unresolved, the 3D models were obtained from AlphaFold prior to ABA docking simulations using AutoDock Vina (ver. 1.1.2) Molecular graphics and analyses were performed with the UCSF Chimera package Chimera was

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