Powerful screening exposes the roles of salicylic acid

DR DANIEL KLESSIG

By identifying the proteins which bind the plant hormone salicylic acid and potentially mediate its myriad physiological effects, **Dr Daniel Klessig** and colleagues are unravelling the complex roles these hormones play in abiotic and biotic plant stresses

What is salicylic acid and how important is it to plant growth and development?

The many responses salicylic acid (SA) treatment induces in plants have been documented for nearly half a century. They include such diverse processes as flowering, stomatal closure, seed germination, adventitious root initiation, thermogenesis, and disease resistance. The roles endogenous SA plays in these processes are not well understood, except for thermogenesis. SA's function in disease resistance has been studied the most intensively, although it is still only partially understood.

Could you describe the two screening methods that you are employing to identify additional salicylic acid-binding proteins (SABPs)?

Our first screen utilises a protein microarray (PMA), in which individual recombinant *Arabidopsis* proteins are printed/attached to a specially-coated microscope slide in a densely packed array of several thousand different proteins per slide. Initially, we incubated this PMA with SA and then tried to identify SA-binding proteins using antibodies that recognise SA. Unfortunately, the extensive washing required to reduce background binding and cross-reactivity led to loss of the bound SA. To overcome this problem, a derivative of SA called 4-azidoSA (4AzSA) is now being used. Ultra violet irradiation of the PMA with bound 4AzSA results in formation of a stable covalent bond (cross-link) between 4AzSA and the protein to which it is bound. The 4AzSA-bound proteins are then identified with anti-SA antibodies.

Our second screen also employs 4AzSA, but in this case it is incubated with a protein extract prepared from *Arabidopsis* leaves. Following UV irradiation, the cross-linked 4AzSA-protein complexes are selected with anti-SA antibodies attached to heavy beads, which enables their separation from the unbound proteins by centrifugation. The enriched 4AzSA-bound proteins are then identified using mass spectroscopy.

Have you made any significant discoveries as a result of using these screening techniques?

To date we have identified more than a dozen new SABPs and even more candidate/putative SABPs, which will require further analysis to determine whether they are true SABPs. A protein is considered a true SABP only if it exhibits SA binding in two or more independent assays. These include cross-linking to 4AzSA, binding radio-labelled SA in a size exclusion chromatography assay, exhibiting altered activity in the presence of SA, and binding to another derivative of SA, 3-aminoethylSA (3AESA), using surface plasmon resonance.

What kind of obstacles have you had to overcome, and what has been the greatest achievement of your work to date?

The biggest challenge of this research has definitely been the development of techniques to identify proteins that bind small molecules. A major problem has been the high level of background, which results in low signal-to-noise ratios and a significant percentage of false positives.

So far, our greatest success has been ameliorating these problems. Going forward, we will focus on elucidating the roles some of these new SABPs play in immunity and other plant processes, as well as characterising the mechanisms through which these proteins are regulated by SA. These findings will allow us to refine our working model of the SA signalling network, which will ultimately be the greatest achievement of our research.

How important is the multidisciplinary approach adopted for characterising SABPs and determining their effects?

Biology is complex. One could argue that plants are even more complex than animals, with the exception of the complex brain of higher mammals. Plants can survive without animals. The reverse is not true; animals need oxygen and fixed carbon (such as sugars and complex carbohydrates) which plants produce through photosynthesis. It is estimated that plants can synthesise approximately 50,000 different compounds, some of which have extraordinary medicinal properties and uses. Animals can produce only a fraction of these. A typical plant in northern climates endures repeated cycles of freezing, extreme heat, drought, nutrient deprivation, radiation, and other abiotic stresses, as well as innumerable biotic stresses caused by infection or predation by pathogens and pests, respectively. To survive, a plant must somehow coordinate and prioritise its responses to these abiotic and biotic environmental assaults and stresses. A typical tree does this for decades, some for centuries, and a few for even millennia. Since SA is known to play important roles in activating defences to both abiotic and biotic stresses, and it also regulates normal plant development, unravelling the complex pathways through which SA mediates its effects will require all the modern tools of genetics, biology, biophysics, biochemistry, and bio-informatics.

Revolutionising the battle against pathogens

Using a variety of novel approaches, **Boyce Thompson Institute** scientists and their collaborators at Cornell University and Columbia University are building a picture of how plants protect themselves against microbial pathogens and the pathways involved in plant defence activation

THERE ARE COUNTLESS processes taking place during the course of plant growth and development. Many of the hormones that are known to affect these processes have been extensively studied, but one that is currently receiving particular attention is salicyclic acid (SA). The role this hormone plays in regulating plant defence responses activated by abiotic and/or biotic stresses is well documented, but SA's mechanism(s) of action is still relatively poorly understood.

Researchers based at Boyce Thompson Institute for Plant Research at Cornell University are keen to shine a spotlight on SA and its functions in plants. They hope to accomplish this by investigating how plants use SA to signal disease resistance following infection by microbial pathogens.

This is a complex topic which requires a comprehensive approach using modern tools derived from genetics, biology, biochemistry, biophysics, chemistry and bio-informatics. For this reason, the team, under the leadership of Dr Daniel Klessig, includes not only biochemists, molecular biologists and geneticists, but also chemists, X-ray crystallographers and bio-informaticists. All of these researchers are collaborating to reach the ultimate goal: to decipher the signal transduction network which leads to activation of plant defences. In particular, they are focusing on elucidating SA-mediated defence signalling.

In addition to elucidating SA's mechanism(s) of action in plants, Klessig and his colleagues anticipate that their results will provide novel insights into how SA functions in animals. For many centuries, plants containing high levels of salicylates, including SA, methyl salicylate, saligenin and their respective glucosides, were known to have important medicinal properties. However, it was not until the 19th Century that the active ingredient in willow bark, salicin, was identified and then later, chemically synthesised as SA. Due to its side effects, SA was subsequently replaced by the synthetic derivative acetyl salicylic acid – commonly known as aspirin – which, from the perspective of many of its users, results in reduced irritation of the gastro-intestinal tract whilst having similar therapeutic outcomes. Today, aspirin is used around the world for pain and fever relief, with the added benefit of being a non-steroidal anti-inflammatory drug. In addition, its prophylactic use is known to reduce the risk of heart attack, stroke, and a number of cancers.

LARGE-SCALE IDENTIFICATION OF BINDING PROTEINS

In the Klessig lab, efforts to elucidate the signalling pathways leading to plant immune responses involve three main projects. The first focuses on investigating the role SA's derivative, methyl salicylate, plays in activating a systemic, broad spectrum, long-lasting resistance termed systemic acquired resistance in *Arabidopsis*, tobacco and potatoes. The second involves characterising CRT1, which is a key component in multiple levels of immunity to a wide range of pathogens, including viruses, bacteria, fungi, and oomycetes. The third focuses on identifying and characterising new SA-binding proteins (SABPs) (Figure 2).

To identify novel SABPs, two new, less proven approaches are being developed that are enabling large-scale identification of proteins that bind to low molecular weight ligands. As a control for these new approaches, concurrent analyses also involve the use of well-established chromatography and biochemical techniques. Klessig and partners anticipate that this strategy can be used to identify proteins that bind other hormones or other low molecular weight ligands in a variety of biological systems, including humans. Furthermore, by elucidating the network of SA signalling circuitry, it might be possible to develop a paradigm for hormone signalling in plants and even animals. "Identification of SABPs in plants and animals will facilitate a between-kingdom comparison of SA effectors/target proteins and mechanism(s) of action for this powerful hormone/drug," asserts Klessig.

USING NATURAL RESISTANCE TO FIGHT DISEASES

One of the major limiting factors for plant-based food production is attack by pathogens. As a result, the agricultural industry relies on a large assortment of chemical inputs that can negatively impact both the economics of farming and the natural environment. In addition, pathogens rapidly develop resistance to these chemicals. Around the world, a significant amount of research effort is being invested into finding more eco-friendly ways to fight crop disease. The problem with many of the available alternatives is that protection is effective only against a select few pathogens, or against specific strains of a particular pathogen. The approach taken by Klessig's team flips this ideology on its head by investigating how a plant's natural defences can be employed in the fight against a wide range of pathogens: "Moreover, this resistance is likely to be sustainable because it results from the cumulative effects of numerous plant defences," he points out.

Since SA is a key hormone that activates natural immunity in plants, identification of novel SABPs should provide important insights into how the SA signal is transduced. In turn, this information may facilitate manipulation of natural resistance, thereby enhancing crop protection. Treating plants with SA has long been known to enhance resistance to a variety of pathogens, but commercialisation of this treatment was impaired by SA's toxicity and limited effectiveness. Several synthetic, resistance-enhancing agents, such as benzothiadiazole, have been identified. Although these compounds are less toxic than SA and

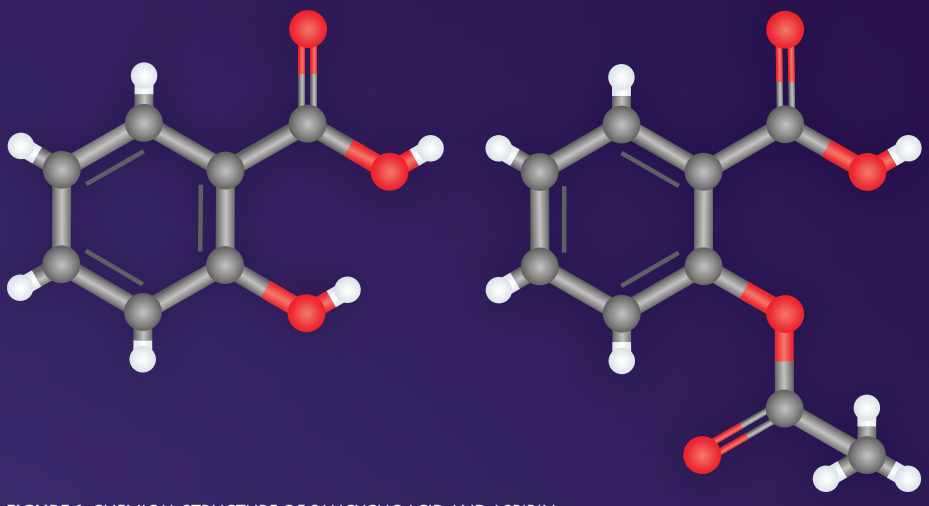

FIGURE 1. CHEMICAL STRUCTURE OF SALICYCLIC ACID AND ASPIRIN

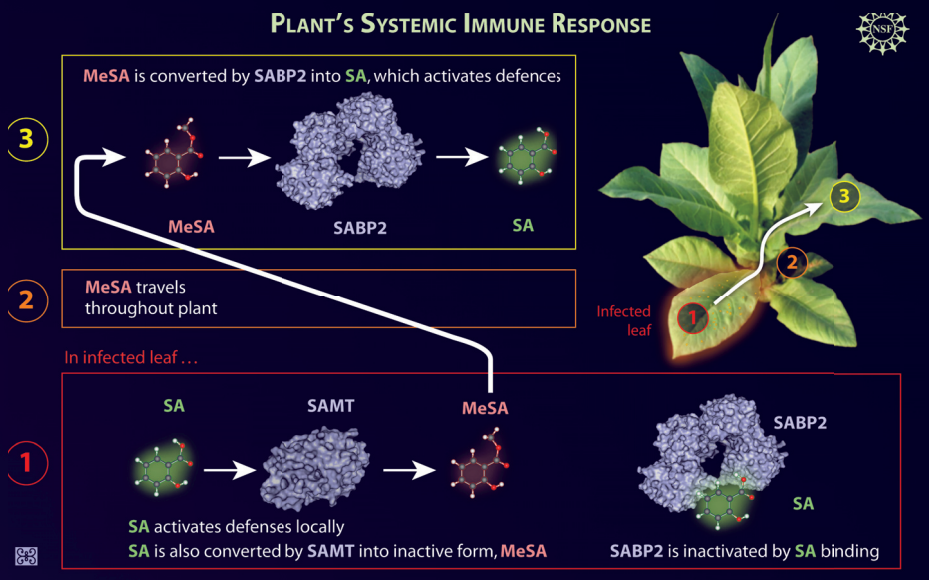

FIGURE 2. Roles of SA methyl transferase (SAMT) and SABP2 in the synthesis and decoding of the long-distance signal for activation of systemic acquired resistance (SAR) methyl salicylate (MeSA).

provide greater protection, their effectiveness can vary depending on the concentration, environmental condition, and pathosystem. Thus, having a detailed knowledge of SA's targets and signalling network should facilitate efforts to identify compounds that maximally induce a plant's defence responses with minimal toxicity. "Optimally we may be able to design and test better 'drugs' for plants, and ultimately animals, that have improved efficacy and safety," comments Klessig.

THE VALUE OF TRANSFERRABLE RESEARCH

Despite over a hundred years of research into aspirin and SA, very few SABPs or other SA targets have been identified in humans, and new mechanisms of SA action continue to be discovered. Klessig and his colleagues are very excited to have identified plant SABPs that may have counterparts in humans. They anticipate that their findings will have an impact on the development of new therapeutic treatments for pain, inflammation, heart disease, stroke, and cancer: "In fact, one of our recently discovered SABPs is known to be involved in immune signalling in humans and is suspected in certain cancers. Interestingly, re-emerging evidence strongly suggests a protective effect of aspirin against one or more of these cancers," Klessig notes. Thus, the strategy developed by this group to identify and characterise plant SABPs may provide exciting insights into how SA functions in both plants and animals.

In addition, the X-ray crystallography and protein modelling studies that have been undertaken as part of this work may make it possible to predict which proteins (regardless of origin) are potential SABPs. "This knowledge," explains Klessig, "could be used to screen the extensive, rapidly expanding protein databanks, including the databank containing all known three-dimensional structures". He argues that this level of knowledge may also help scientists and healthcare professionals to predict unwanted side effects of commonly-used drugs like aspirin.

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INTELLIGENCE

THE ARABIDOPSIS SALICYLIC ACID SIGNALING NETWORK: A PARADIGM FOR PHYTOHORMONE SIGNALING

OBJECTIVES

- To obtain insights into how SA carries out its varied functions, two powerful high throughput screens are being developed to identify new SA-binding proteins
- To facilitate the development of high throughput screens for the large-scale identification of proteins that bind small molecules (like hormones); these could be used for the characterisation of other hormone or small molecule signalling networks in animals, as well as plants

PARTNERS

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