Determining the fate of proteins

Professor Renier van der Hoorn reveals why he has devoted his professional life to understanding how proteases regulate plant proteins, and discusses the partnership possibilities his research offers

What first sparked your interest in plant science?

Plant science has fascinated me since early childhood. I grew up with my father's tropical greenhouse, where he grew millions of decorative plants for the EU market. My desire to understand the manipulation of plants by microbes propelled me through chemistry and biology lectures and feeds my endless curiosity in understanding the underlying molecular mechanisms of plant manipulation.

From investigations into the wild tobacco (*Nicotiana benthamiana*)-*Pseudomonas syringae* model system, what have you been able to infer about apoplast manipulation on commodity crops?

Our studies revealed that probably every leaf pathogen manipulates the apoplast (extracellular space). All of these pathogens are exposed to hydrolytic enzymes secreted by the host during infection. Having realised their importance, we were able to design strategies that make hydrolases more effective; for example, by making them insensitive to manipulation by pathogen-derived inhibitors. Our findings are directly applicable because wild tobacco is a close relative of important crop plants like potato, tomato and pepper.

Can you explain the term 'molecular pharming'? Which factors have led to its growth in popularity?

Molecular pharming is the production of recombinant proteins in plants for medicinal purposes. Free of animal pathogens, plant-based production systems are safer to use. Depending on the product, they can also be cheaper than other platforms. Over the past few years, the field has advanced tremendously from the introduction and improvement of transient expression systems. These are often based on the infiltration of leaves with modified *Agrobacterium tumefaciens*, which transfers genes of interest into the plant cell. In combination with virus-based amplification, silencing inhibitors and efficient translation, recombinant proteins can be produced within a few days with levels up to 50 per cent of the total proteome. This expression system can be faster, less expensive and more flexible than any other existing protein expression system.

What is the potential of producing recombinant proteins in *N. benthamiana*? Are there any associated problems and if so, how do you seek to solve them?

Molecular pharming by transient protein expression in *N. benthamiana* has great potential. Companies like Medicago use this system to produce 10 million vaccines against influenza every month. Most proteins, however, are not stable in this expression system as they are processed and degraded by plant proteases, many of which are induced by agroinfiltration. The aim of our European Research Council (ERC)-funded *GreenProteases* project is to deplete the proteases that process recombinant proteins. This project will also lead to tools that will identify the natural substrates of these proteases and determine their role in immunity.

The chemistry of plant biology

The Plant Chemetics Laboratory at the **University of Oxford** provides an ideal environment for collaboration on developing and testing new chemistry-based approaches to plant biology research

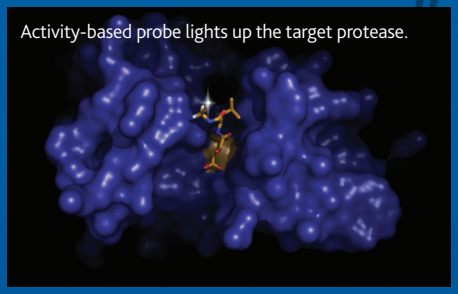
OF THE MAJOR scientific disciplines, plant science is generally associated with biology. However, as genetic investigations grow ever more complex, chemistry can play an increasingly important role. The University of Oxford's Plant Chemetics Laboratory is at the forefront of developing and applying novel chemistry tools to address fundamental questions in plant biology. "Chemetics is a contraction of chemical genetics," explains Laboratory Head, Professor Renier van der Hoorn. "We believe it is possible to use chemicals to unravel protein functions. The use of chemical tools to address biological questions is very important in my line of work."

The Lab has pioneered the development of activity-based proteomics in plant science; which is the driving force behind the team's work on understanding apoplast manipulation and its proteolytic machinery in order to improve molecular pharming methods. The researchers collaborate extensively with institutes and businesses, and are keen to promote their work across a diverse range of disciplines.

DISPLAYING THE ACTIVE PROTEOME

In 2003, the Plant Chemetics Lab was the first to apply activity-based protein profiling (ABPP) to plant science. The benefit of this technology over traditional enzyme assays is that it displays the active proteome in its natural environment, without any pre-purification, thus facilitating study into the effect of regulation by cofactors and interacting proteins. The assay displays the availability and reactivity of active sites rather than the substrate conversion that traditional assays monitor – a hallmark for protein activity. "The dynamics of proteins are difficult to predict from their presence as their activity is predominantly regulated by various post-translational processes," van der Hoorn elucidates. "Technologies capable of revealing the activities of proteomes are therefore crucial to uncover an important layer of information in biological processes."

Protein activities in proteomes are displayed using tagged small molecules or probes – biotinylated or fluorescent class-specific



Activity-based probe lights up the target protease.

inhibitors that react with the active sites of enzymes in an activity-dependent manner. The relevance of detected enzyme activities are examined using traditional reverse genetics and modern targeted chemical genetics. A covalent, irreversible bond between the enzyme and the labelled inhibitor is the result of this reaction, enabling subsequent analysis. Labelled proteins can be purified and detected on western blots, and their identity determined by mass spectrometry. "We are the first to introduce new probes, fluorescent profiling, labelling site identification and quantitative methods," enthuses van der Hoorn. "Moreover, we have discovered hydrolase manipulation that was not predicted based on transcript or protein levels."

The Lab's work introducing ABPP in plant science has resulted in a large and unique probe library and extensive knowledge on enzyme profiling. "By collaborating with most ABPP pioneers in medical science, we have the largest collection of activity-based probes in the world," van der

Infiltration of bacteria for infection and recombinant protein production.

Your research themes coalesce in the characterisation of proteases. Why is this significant?

Proteases can activate and deactivate other proteins, thereby determining their fate. Some are highly specific while others have a very broad substrate range. However, despite the fact that all proteins are regulated by proteases, of which plants produce hundreds, we do not know their control mechanisms. This is partly because protein processing is hard to predict as it depends on 3D structure and post-translational modifications. Protease research is challenging as it requires excellent genetic, chemical and proteomic tools. Not many researchers are active in this area because it requires strong interdisciplinary collaboration.

How important is outreach to your research? Are there opportunities for visiting scientists?

Outreach is very important – our techniques offer an excellent collaboration platform and I am also interested in other research fields. We have received over 35 visiting scientists in the past few years. Most join for two to three weeks to investigate their proteomes with our probes and protocols. This not only brings novel insights in protein regulation, but also creates a very dynamic atmosphere in the lab.

To what extent do you work with industry?

I am very keen to engage more in collaboration with industrial partners. Working with seed companies, we discovered that our techniques can predict seed quality. We can also display targets of agrochemicals and reveal the active proteome during industrial processes such as beer brewing or food processing. There are tremendous opportunities to collaborate with industry using our procedures.

PLANT CHEMETICS LABORATORY

OBJECTIVES

To expand activity-based protein profiling (ABPP) in life sciences and apply it through collaborations. In addition, to understand and exploit apoplast manipulation by microbes to identify new crop protection strategies and control extracellular protein processing in plants to improve molecular pharming.

KEY COLLABORATORS

ABPP development: **Ben Cravatt**, Scripps Institute • **Matt Bogyo**, Stanford Medical School • **Hermen Overkleeft**, Leiden University • **Markus Kaiser**, Duisburg-Essen University • **Stephan Sieber**, LMU Munich • **Steven Verhelst**, TUM Munich • **Eranthie Weerapana**, Boston College • **Benedikt Kessler**, Oxford University • **Galia Blum**, Hebrew University • **Matthew Pattricelli**, ActivX

ABPP application: **Dorothea Bartels**, Bonn University • **Gunther Doehlemann**, MPI Marburg • **Hannele Tuominen**, Umea University • **Mercedes Royuela**, Pamplona University • **Christiane Funk**, Umea University • **Florian Grundler**, Bonn University

Plant-microbe interactions: **Sophien Kamoun**, Sainsbury lab • **Matthieu Joosten**, Wageningen University • **Jim Alfano**, Nebraska University • **Robert Dudler**, Zurich University

Molecular Pharming: **Andreas Schiermeier**, Fraunhofer Aachen • **Lukas Mach**, BOKU Vienna • **Rita Abranches**, ITQB

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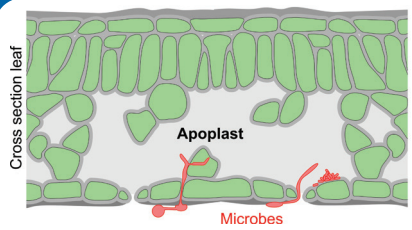
RENIER VAN DER HOORN studied chemistry in Leiden and completed his PhD study in 2001 at Wageningen University. He initiated his own lab in 2005 at the Max Planck Institute in Cologne. Since early 2014 he has held the role of Associate Professor at Oxford University.



Microbes colonise the plant extracellular space (apoplast).

Hoorn proclaims. "This has attracted a flood of visiting scientists who come to test these probes and protocols on their chosen biological system." The researchers can now monitor the activities of over 3,000 plant proteins and are introducing new probes for other protein classes and improved analytics. ABPP technology is rapidly expanding and has enormous potential; it can be applied to any organism and most enzyme classes, as well as living cells, tissues and whole organisms. It therefore offers opportunities for a broad range of collaborations on subjects including seed quality, herbicides and fermentation processes, as well as the production of recombinant proteins, control over plant diseases and structural biology.

APOPLAST MANIPULATION

A major programme the Lab is currently working on utilises ABPP to study the manipulation of apoplasts by plant pathogens. "We chose to work on plant-pathogen interactions, since these are likely to encompass a fascinating molecular battleground where two organisms interfere in each other's protein activities," clarifies van der Hoorn. "This field of research is large and unexplored as most research focuses on understanding plant immunity."

> It is anticipated that the apoplast manipulation project will lead to better insight into how the defence response of plants can be improved to increase pathogen resistance

Arguably the largest biological interface on Earth, the extracellular space in plants – the apoplast – is heavily colonised by microbes, mediating all that passes between plants and their environment. Host plants respond to these microbes by secreting defence compounds and proteins; including a broad range of hydrolytic enzymes, which are potentially harmful to the microbes that inhabit the apoplast. The research is based on the hypothesis that successful pathogens are able to manipulate these hydrolytic enzymes to suppress their activity, and thus infect the plant. The Lab aims to identify which enzyme activities have been manipulated in order to unravel their molecular mechanism and biological relevance.

So far, in using a model system between the interaction of wild tobacco (*Nicotiana benthamiana*) and commensal and pathogenic strains of the bacterium *Pseudomonas syringae*. Infection of other Solanaceae plants by the leaf mould fungus *Cladosporium fulvum* and the potato blight pathogen *Phytophthora infestans* have also been investigated. By studying tomato proteases, the team has also revealed how important host enzymes are in basal defence, and how the co-evolutionary arms race has shaped natural variation in host proteases.

It is anticipated that the apoplast manipulation project will lead to better insight into how the defence response of plants can be improved to increase pathogen resistance. One such approach is to modify plant proteins such that they become insensitive to pathogen manipulation, offering a strong opportunity for collaboration. In addition, van der Hoorn is also intrigued by the molecular details of manipulative interactions in the apoplast and as such, is seeking new collaborations in order to resolve crystal structures of protease-inhibitor complexes at the plant-pathogen interface.

CONTROLLING THE PROTEOLYTIC MACHINERY

More recently, the Plant Chemetics group has begun a European Research Council (ERC)-funded research programme aimed at unravelling the apoplast proteolytic machinery, particularly of agroinfiltrated *N. benthamiana*, to increase recombinant glycoprotein production and identify the biological roles of secreted proteases in plants. Plants release dozens of these enzymes that shape the extracellular proteome, but their biological roles and substrates are poorly understood. This knowledge is essential to overcome crucial problems in the biotech industry. The expression system used in molecular pharming to produce proteins in plants – agroinfiltration – has become the standard way to produce influenza vaccines. However, this induces immune responses and thus the secretion of proteases. These enzymes could play crucial roles in the degradation of recombinant glycoproteins.

The project could have wide-reaching applications, as van der Hoorn makes clear: "I imagine that in a few years companies will be interested in testing the production of their chosen recombinant glycoproteins under protease-depleted conditions. This could result in vaccines against infectious livestock diseases, bioterror agents or threatening human diseases in developing countries". Production of antibodies and glycohormones could be possible if plant proteolytic machinery can be controlled, and with industry support this may soon be a reality.