Molecular Interactions of Endophytic Actinobacteria in Wheat and Arabidopsis

A thesis submitted for the award
Doctor of Philosophy
at Flinders University

Vanessa Michelle Conn

Department of Medical Biotechnology
School of Medicine, Faculty of Health Sciences,
Flinders University

2005

Declarations

I certify that this thesis does not contain material which had been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes.

Vanessa Michelle Conn

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Acknowledgments

First and foremost I want to thank my wonderful husband, Simon, for all the love and support he has given me. Thank you for all your constant encouragement and listening to me talk about my work all the time. I love working with you but it is even better knowning you will be at home with me too!

I would like to give a big thank you to my prinicipal supervisor Professor Chris Franco who has taught me a lot during my PhD but it has always been fun. You have pushed me to achieve more with my studies and for that I am especially grateful. I would also like to thank my supervisor Dr. Mandy Walker for her invaluable advice with the Arabidopsis experiments but also for her unending support, encourgament and guidance. Also, my thanks go to Dr. Margaret Roper for her words of encouragement and for proofreading this epic! Thank you also to the GRDC for awarding me a PhD scholarship and a travel grant to present my work.

Thank you to Dr. Margaret McCully and Dr. Cheng Huang for the expert assistance with the cryo-scanning electron microscopy and Dr. Kerry Gascoigne for the scanning electron microscopy.

Thank you to the Department of Medical Biotechnology for putting up with me and my signs. I would especially like to thank Angela Binns for her tireless work in the laboratory; it has been a pleasure working with you. Lian and Kushari who made my first year incredibly fun, there is nothing better than listening to Madonna while you work, I am the material girl. Chris Curtin I would also like to thank you for suffering through endless RNA extractions with me but also for the help with real-time PCR and for letting me vent my frustrations, for that alone you deserve a whole

chapter dedicated to you!!! Thank you to Phil for being a great colleague over these years, but I will have to say I will not miss the smell of those boxes! Nigel, it has been fun working with you but I now pass the RNA extraction baton to you, enjoy!

Finally I would like to thank all my family and friends who have supported me throughout my studies, and yes I have finally finished.

Abbreviations

µl; ml; l: microlitre; millilitre; litre

ρM; µM; mM; M: picomolar; micromolar; millimolar; molar

½ MS salts: half strength Murashige and Skoog salt medium

ACC: 1-aminocyclopropane-1-carboxylic acid

AM fungi: arbuscular mycorrhizal fungi

Ap: abundance percentage

AUD: Australian dollar

bp: base pairs

CFU: colony forming units

CR: crown rot

CTAB: cetryltrimethylammonium bromide

DGGE: denaturing gradient gel electrophoresis

DNA: deoxyribonucleic acid

dNTPs: dinucleotide triphosphates

Ecc: *Erwinia carotovora* subsp. *carotovora*

EDTA: ethylenediamine tetraacetic acid

ET: ethylene

eGFP: enhanced green fluorescent protein

FAME: fatty acid methyl ester

FHB: Fusarium head blight

Ggt: *Gaeumannomyces graminis* var. *tritici*

GRDC: Grains Research and Development Corporation

HEX: hexachlorofluorescein phosphoramidite

hr: hour

hrs: hours

HR: hypersensitive response

IPTG: isopropyl β-D-thiogalactoside

ISR: induced systemic resistance

JA: jasmonic acid

LB: Luria broth

MeJA: methyl jasmonate

min: minutes

MS: mannitol soy agar

NCBI: National Centre for Biotechnology Information

ng; μg; mg; kg: nanograms; micrograms; milligrams; kilograms

NSW: New South Wales

PCR: polymerase chain reaction

PDA: potato dextrose agar

PGPR: plant growth promoting rhizobacteria

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

RO: reverse osmosis

RT: room temperature

SA: salicylic acid

SAR: systemic acquired resistance

SDS: sodium dodecyl sulphate

sp.: species (singular)

spp.: species (plural)

TBE: tris-borate EDTA

TET: 6-carboxy-2',4,7,7'-tetrachlorofluorescein

TGGE: temperature gradient gel electrophoresis

T-RFLP: terminal restriction fragment length polymorphism

TRF: terminal restriction fragment

UV: ultraviolet

YME: yeast malt extract agar

Abstract

Wheat is the most economically important crop forming one quarter of Australian farm production. The wheat industry is severely affected by diseases, with fungal pathogens causing the most important economic losses in Australia. The application of fungicides and chemicals can control crop diseases to a certain extent, however, it is expensive and public concern for the environment has led to alternative methods of disease control to be sought, including the use of microorganisms as biological control agents. Microorganisms are abundant in the soil adjacent to plant roots (rhizosphere) and within healthy plant tissue (endophytic) and a proportion possess plant growth promotion and disease resistance properties.

Actinobacteria are gram-positive, filamentous bacteria capable of secondary metabolite production such as antibiotics and antifungal compounds. A number of the biologically active endophytes belonging to the Actinobacteria phylum were isolated in our laboratory. A number of these isolates were capable of suppressing the wheat fungal pathogens *Rhizoctonia solani*, *Pythium* sp. and *Gaeumannomyces graminis* var. *tritici,* both *in vitro* and *in planta* indicating the potential for the actinobacteria to be used as biocontrol agents. The aim of this research was to investigate the molecular mechanisms underlying this plant-microbe interaction.

The indigenous microbial populations present in the rhizosphere and endophytic environment are critical to plant health and disruptions of these populations are detrimental. The culture-independent technique Terminal Restriction Fragment Length Polymorphism (T-RFLP) was used to characterise the endophytic actinobacteria population of wheat roots under different conditions. Soils which support a higher number of indigenous microorganisms result in wheat roots with higher endophytic actinobacterial diversity and level of colonisation. Sequencing of 16S rRNA gene clones, obtained using the same actinobacteria-biased PCR primers that were used in the T-RFLP analysis, confirmed the presence of the actinobacterial diversity, and identified a number of *Mycobacterium* and *Streptomyces* species. It was found that the endophytic actinobacterial population of the wheat plants contained a higher diversity of endophytic actinobacteria than reported previously, and that this diversity varied significantly among different field soils.

The endophytic actinobacteria have previously been shown to protect wheat from disease and enhance growth when coated onto the seed before sowing. As the endophytes isolated were recognised as potential biocontrol agents, the impact on the indigenous endophytic microbial population was investigated. Utilising the T-RFLP technique it was established that the use of a commercial microbial inoculant, containing a large number of soil bacterial and fungal strains applied to the soil, disrupts the indigenous endophyte population present in the wheat roots. The hypothesis is that non-indigenous microbes proliferate and dominate in the soil preventing a number of endophytic-competent actinobacterial genera from access to the seed and ultimately endophytic colonisation of the wheat roots. This dramatically reduces diversity of endophytes and level of colonisation. In contrast the use of a single endophytic actinobacteria endophyte inoculant results in a 3-fold increase in colonisation by the added inoculant, but does not significantly affect this indigenous

population.

Colonisation of healthy plant tissues with fungal endophytes has been shown to improve the competitive fitness with enhanced tolerance to abiotic and biotic stress and improved resistance to pathogens and herbivores. In this study the fungal endophyte population of wheat plants grown in four different soils was analysed using partial sequencing of 18S rRNA gene sequences. Sequence anlaysis of clones revealed a diverse range of fungal endophytes. In this diverse range of fungal endophytes a number sequences were highly similar to those of previously known fungal phytopathogens. A number of sequences detected were similar to fungal species previously identified in soil or plant material but not as endophytes. The remaining sequences were similar to fungal species without a known relationship with plants.

Plants have developed an inducible mechanism of defence against pathogens. In addition to local responses plants have developed a mechanism to protect uninfected tissue through a signal that spreads systemically inducing changes in gene expression. In the model plant *Arabidopsis thaliana* activation of the Systemic Acquired Resistance (SAR) pathway and the Jasmonate (JA)/Ethylene (ET) pathway is characterised by the production of pathogenesis-related (PR) and antimicrobial proteins resulting in systemic pathogen resistance. Endophytic actinobacteria, isolated from healthy wheat roots in our laboratory, have been shown to enhance disease resistance to multiple pathogens in wheat when coated onto the seed before sowing. Real Time RT-PCR was used to determine if key genes in the SAR and JA/ET pathways were induced in response to inoculation with endophytic actinobacteria.

Inoculation of wild-type *Arabidopsis thaliana* with selected strains of endophytic actinobacteria was able to 'prime' the defence pathways by inducing low level expression of SAR and JA/ET genes. Upon pathogen infection the defence-genes are strongly up-regulated and the endophyte coated plants had significantly higher expression of these genes compared to un-inoculated plants. Resistance to the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* was mediated by the JA/ET pathway whereas the fungal pathogen *Fusarium oxysporum* triggered primarily the SAR pathway.

Further analysis of the endophytic actinobacteria-mediated resistance was performed using the *Streptomyces* sp. EN27 and Arabidopsis defence-compromised mutants. It was found that resistance to *E. carotovora* subsp. *carotovora* mediated by *Streptomyces* sp. EN27 occurred via a NPR1-independent pathway and required salicylic acid whereas the jasmonic acid and ethylene signalling molecules were not essential. In contrast resistance to *F. oxysporum* mediated by *Streptomyces* sp. EN27 occurred via a NPR1-dependent pathway but also required salicylic acid and was JA- and ET-independent.

This research demonstrated that inoculating wheat with endophytic actinobacteria does not disrupt the indigenous endophytic population and may be inducing systemic resistance by activating defence pathways which lead to the expression of antimicrobial genes and resistance to a broad range of pathogens.