Sensing and signalling mechanical stress during intercalary growth in *Epichloë* grass endophytes

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Kahandawa Geeganaarachchige Sameera
Upanada Ariyawansa
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Abstract

*Epichloë festuc*ae is an agronomically important seed-transmitted endophytic fungus that grows symbiotically within the intercellular spaces of temperate grass species. This fungus has previously been shown to undergo hyphal intercalary growth during host leaf colonization, a highly unusual mechanism of division and extension in non-apical compartments in vegetative hyphae, as an adaptation to colonise rapidly elongating host cells in the developing leaf. However the exact mechanism that triggers intercalary growth was not known. In this study I aimed to test the hypothesis that intercalary growth is stimulated by mechanical stretch imposed by attachment of hyphae to elongating host cells, and that this stress is sensed by mechano-sensors located on hyphal membranes.

To test this hypothesis a novel technique was designed and optimised to stretch fungal hyphae under *in vitro* conditions. Investigation of un-stretched hyphae showed that d*e novo* compartmentalization occurs in sub-apical compartments of *E. festucae* hyphae according to a compartment length-dependent hierarchy. Subjecting these sub-apical compartments to mechanical stretching showed that hyphal compartment lengths can be increased while maintaining viability, provided that the stretch is within tolerable limits. It further showed that the stretched compartments undergo *de novo* compartmentalization (nuclear division and septation) similar to un-stretched hyphae but at a significantly higher rate, fulfilling the basic requirements for intercalary growth.

E. festucae WscA and MidA, which are orthologues of a yeast cell wall stress and a stretch-activated calcium channel protein respectively, were functionally characterized in order to test the possible involvement of these mechano-sensors in intercalary

growth. Their roles in general hyphal apical growth, cell wall construction and integrity maintenance during growth in culture were confirmed. The limited ability of Δ*midA* mutants to colonise developing leaves indicated a possible role in intercalary growth, while Δ*wscA* mutants showed wild-type levels of host colonization. In future, the Δ*midA* and Δ*wscA* mutants will be subjected to mechanical stretch *in vitro* to further understand their roles in mechano-sensing and intercalary growth.

Given the possible involvement of the stretch-activated calcium channel MidA in intercalary growth, a successful technique was developed to study calcium signalling and distribution in *E. festucae* using the genetically-encoded calcium sensor GCaMP5. Investigations revealed the presence of MidA-driven Ca^{2+} pulses confined to the hyphal tips with unique signatures of temporal and spatial dynamics generated by influx of Ca^{2+}. The presence of active sub-apical Ca^{2+} uptake systems were confirmed, manifested as occasional Ca^{2+} pulses in sub-apical compartments that seemed to increase in frequency with mechanical perturbation, indicating a potential crucial role in mechanical stress-driven intercalary growth.

In conclusion a prospective model for intercalary growth in the leaf expansion zone is proposed. Mechanical stretching of hyphae results in increased compartment lengths, accompanied by compartmentalization in sub-apical compartments that allows hyphae to extend along their length. Membrane distortion due to stretching activates MidA, triggering a calcium signalling cascade to stimulate cell wall synthesis and other cellular processes.

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Abbreviations

aa	Amino acid
Amp^R	Ampicillin resistant
ATP	Adenosine triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
BS	Blocking solution
CaM	Calmodulin
$Chlo^R$	chloramphenicol resistant
cm	Centimeter
CR	Congo red
CW	Calcofluor White
CWI	Cell wall integrity
DIC	Differential interference contrast
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
E value	Expect value
EGFP	Enhanced Green Fluorescent Protein

EGTA	Ethylene glycol-bis(2aminoethylether)-N,N,N′,N′-tetraacetic acid
ER	Endoplasmic reticulum
fmole	Femtomole
FRET	Förster resonance energy transfer
g	Gram
GECI	Genetically encoded calcium indicator
Gen^R	Geniticin resistant
GS	1,3-β-glucan synthase complex
GTP	Guanosine triphosphate
h	Hour(s)
HACS	High affinity calcium system
Hyg^R	Hygromycin resistant
Kb	Kilobase(s)
Kan^R	Kanamycin resistant
L.S	Longitudinal section
LACS	Low-affinity calcium system
LB	Luria-Bertani
LBA	Luria-Bertani agar
M	Molar
m/s	Meters per second
MAPK	Mitogen-activated protein kinases
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase

mg	Milligram
min	Minutes
mm	Millimeter
mM	Millimole
NCBI	National Center for Biotechnology Information
NCM	Nitrocellulose membrane
ng	Nanograms
nM	Nanomole
nm	Nanometers
^{o}C	Degrees Celsius
ORF	Open reading frame
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PEG	Polyethylene glycol
PKC	Protein kinase C
rcf	Relative centrifugal force
RE	Restriction enzyme
RG	Regeneration
rpm	Revolutions per minute
RT	Room temperature
s	Seconds
SAM	Shoot apical meristem

SD	Standard deviation
SDS	Sodium dodecyl sulphate
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptors
Sorb	Sorbitol
T.S	Transverse section
TAE	Tris-acetate-EDTA
tBLASTn	Translated nucleotide database search using a protein query
TEF	Translation elongation factor
U	Unit
USA	United States of America
v/v	Volume/volume ratio
w/v	Weight/volume ratio
WT	Wild type
YFP	Yellow fluorescent Protein
YH2A	Histone protein HH2A fused to yellow fluorescent protein
$[Ca^{2+}]_c$	Cytoplasmic calcium concentration
µg	Microgram
µL	Microliters
µm	Micrometer
µM	Micromolar