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<th>Title</th>
<th>Ceratopteris richardii (C-Fern): a model for investigating adaptive modification of vascular plant cell walls</th>
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INTRODUCTION

Driven by an increased awareness of the impact of plant cell wall composition on environmental responses, and their commercial exploitation, as well as by curiosity, and facilitated by technological developments, cell wall diversity and evolution has increasingly become a major research focus in the last 5 years (Popper, 2008; Sarkar et al., 2009; Yin et al., 2009; Popper and Tuohy, 2010; Sørensen et al., 2010; Popper et al., 2011; Fangel et al., 2012). Cell walls are involved at every level of plant morphology, growth and development, and have changed during evolution (Popper and Fry, 2004; Sørensen et al., 2010; Popper et al., 2011; Fangel et al., 2012); the evolution of morpho-anatomical characters in particular relies on cell wall modification. Cell division, cell expansion and cell differentiation, which give rise to the generation of cell shape and plant form, are intrinsically cell wall processes (Szymanski and Cosgrove, 2009; Boudaoud, 2010). For example plant cell division necessitates coordinated synthesis and deposition of a new wall between the two daughter cells and turgor-driven cell expansion depends on wall relaxation mediated for example by enzymes, such as xyloglucan endotransglycosylase (Fry et al., 1992; Nishitani and Tominaga, 1992), or proteins, such as expansins (McQueen-Mason et al., 1992; McQueen-Mason and Cosgrove, 1995), whose presence and action is dependent on wall composition (Frankovich and Fry, 2011).

Although initially highlighted by biochemical analyses understanding of the taxonomically-based diversity of plant and algal cell wall components and their biosynthesis has been revolutionized by the availability of sequenced plant genomes (Yin et al., 2009; Popper et al., 2011). There are currently around forty fully sequenced plant and algal genomes (Goodstein et al., 2012). However, in sharp contrast to the late nineteenth century pteridomania which endangered some species (Dyer et al., 2001) ferns (here delimited as the monilophytes which includes ferns, whisk ferns and horsetails) now receive comparatively less attention than many other plant groups and full sequences of fern genomes are, as yet, unavailable (Barker and Wolf, 2010). Cronk (2009) noted that early genome sequencing focused heavily on angiosperms; perhaps unsurprisingly given their economic prominence. Recently the need for greater phylogenetic coverage has been recognized and, aided by technological advances, has led to the sequencing of representatives of algae and earlier diverged land plants with phylogenetic significance and possessing relatively small genomes including the green alga, Ostreococcus tauri (Durell et al., 2006) and the spike moss, Selaginella moellendorffii (Banks et al., 2011). Despite being hampered by its exceptionally large genome size (Burleigh et al., 2012) at ~150 times greater than that of Arabidopsis, the first gymnosperm genome, Norway spruce (Picea abies) was published earlier this year (Nystedt et al., 2013).

Thus, the remaining gaps include sequenced genomes of a fern (Nakazato et al., 2006) and a hornwort (Cronk, 2009). Similarly to gymnosperms, ferns include plants of significant commercial, economic and ecological value such as the aquatic giant salvinia (Salvinia molesta) that was recently added to the International Union for Conservation of Nature (IUCN) worst invasive alien species list (Luque et al., 2013). Ferns have a worldwide distribution and are adapted to diverse habitats, often occurring as pioneer...
species and occasionally becoming ecologically dominant e.g.,
Phleum pratense (commonly known as bracken). Additionally,
although ferns consist of ~15,000 species and therefore comprise
only around 3% of vascular plant diversity globally (Schachtel
and Pryer, 2008) they may account for up to 20% of vascular plant
diversity in areas such as the West Indies (Groombridge, 1992).

Given the ecological importance and placement of ferns as easily
diverging euphlyophytes (a sub-division of vascular plants including
monilophytes and seed plants) a better understanding of their
cell wall complexity, in terms of composition, biosynthesis and
tissue- and cell-specific variation, may provide novel insight into
key developmental processes, for example vascularisation of leaves
(Cronk, 2009), as well as providing unique opportunity to investi-
gate gametophyte-specific processes. In this perspective we review
the current state of knowledge regarding fern cell wall composi-
tion, the impact of genome sequencing on our understanding of
evolutionary pathways of cell wall biosynthetic genes, the require-
ment for a sequenced fern genome and how this might impact
future research focused on plant cell wall biology, physiology,
evolution and development.

**FERN CELL WALLS**

Biochemical analyses have contributed much of what we know
about fern cell walls and indicate that they are compositionally
similar, though not identical, to those of flowering plants. More
specifically, mannose-containing polysaccharides such as man-
nan and glucomannan appear to be abundant in ferns, whereas
pectins appear to be present in lower concentrations than found
in other plants (Popper and Fry, 2004; Silva et al., 2011). On the
other hand, some wall components have a structure and function
which appears to pre-date the divergence of ferns from gym-
nosperms and flowering plants. α-Expansins, wall-acting proteins
which mediate acid-induced wall creep (McQueen-Mason et al.,
1992; McQueen-Mason and Cosgrove, 1995), have not only been
identified from the ferns Marsilea quadrifolia and Regnellodium
diphyllum (both species of aquatic ferns) by their homology to
flowering plant α-expansins but protein extracts from M. quadri-
folia are capable of inducing wall creep in cucumber cell walls
(Kim et al., 2000). The importance of cell wall composition and
metabolism to plants environmental responses and survival, as
well as our exploitation of them, deem cell wall composition worthy
of extensive exploration. Current approaches include application
of specific cell wall-directed tools and methodologies (Fry, 2000;
Popper, 2011) including carbohydrate microarrays (Moller et al.,
2007), glycome profiling (Parathil et al., 2012) and microscopy
utilizing wall-directed monoclonal antibodies (mAbs), as exem-
plified in Figure 1 (right hand side), and carbohydrate-binding
modules (CBMs; Sorensen et al., 2009; Patautil et al., 2011; Hervé
et al., 2011) as well as comparative genome analysis.

**THE LYCOPHYTE-EUPHYLLOPHYTE DIVIDE**

The genes responsible for the biosynthesis of plant cell wall com-
ponents are increasingly well identified and characterized. However,
the genes responsible for the synthesis and metabolism of some
components are not yet fully elucidated (Harbolt et al., 2012).
This is particularly true for seemingly anomalous occurrences
of specific wall components. For instance, although cellulose
synthase-like (CSSL) supergene family members CefPs and CefTs
are responsible for synthesizing β-(1,3),(1,4)-glucan (mixed link-
age glucan, MLG) in members of the Psalid (grasses; Richmond
and Somerville, 2000), Burton et al., 2006, 2008; Dublin et al., 2009)
the absence of orthologues of these genes (Harbolt et al., 2012)
confounds detection of MLG in Selaginella moellendorffii and syn-
thesis of MLG in this plant remains enigmatic but is corroborated
by the discovery of MLG in Equisetum (horsetails; Fry et al., 2008;
Sorensen et al., 2008).

Sequencing and genome analysis of the whisk fern Selaginella
moellendorffii, chosen for its small genome size (Banks et al.,
2011; Harbolt et al., 2012), has already proven invaluable to elucidat-
ing diversification of cell wall components and their biosynthetic
machinery (Popper et al., 2011). Lycophytes are the earliest diverg-
ing extant plants to have a vascular system and a dominant spor-
ophyte generation. However, disparities in genome sequence and
cell wall biochemistry between Selaginella and other sequenced
vascular plants including Arabidopsis (Arabidopsis Genome
Initiative, 2000), Populus (Tuexkan et al., 2006), and the grasses, rice
(International Rice Sequencing Project, 2005), and Brachypodium
(International Brachypodium Initiative, 2010), detailed below,
highlight the need for fern sequences and detailed cell wall studies,
not only to help better understand ferns, but also euphlyophyte
evolution and development.

Although the majority of cell wall components found in flower-
ing plants also occur in Selaginella, Harbolt et al. (2012) observed
differences in the abundance, localization and extractability
between wall polymers in flowering plants compared with those in
Selaginella. This is potentially indicative of differences in interac-
tions between specific cell wall components. Pectins in particular
appeared to not only be more abundant in lycophytes than in
angiosperms but also required harsher extraction procedures
(Harbolt et al., 2012). The pectin, rhamnogalacturonan II, was
found to occur in all vascular plant groups in similar concentra-
tion but, despite appearing to be highly conserved, exhibited a minor
compositional variance, in lycophytes, ferns and whisk ferns; a
rhamnose residue was replaced by a 3-O-methyl rhamnose residue
in one of the side chains (Matsunaga et al., 2004). Furthermore,
some cell wall features appear to have arisen through convergent
evolution. For example the regulation and biosynthesis of syringyl
(S) lignin which reinforces the secondary cell walls in the vascular
tissue of flowering plants and lycophytes, but is absent from the
majority of ferns and gymnosperms, occur via independent path-
ways (Weng et al., 2008, 2011; Zhao et al., 2010; Novo-Uzal et al.,
2012). In angiosperms S lignin is synthesized from guaiacyl lignin
intermediates by ferulic acid/ferulaldehyde/ferulic alcohol
5-hydroxylase (F5H) and although Selaginella moellendorffii con-
tains a functional F5H it is not orthologous to angiosperm F5Hs
instead belonging to a clade of genes unique to Selaginella (Weng
et al., 2008). As Harbolt et al. (2012) point out this is in direct
contrast with an apparent lack of diversification and specializa-
tion within the cellulase synthase (CESA) superfamily. Homologs
of IRX10, also involved in vascular formation in land plants, were
found in the moss Physcomitrella patens and were recently reported
to exhibit functional conservation with those from Arabidopsis
(Hornblad et al., 2013). Taken together these data suggest that at
least some components of vascular tissues considered to be a
Gametophytes develop as hermaphrodites or males. Sporophyte fronds are dimorphic. Fronds are initially sterile and oval shaped to three-lobed but new fronds become progressively larger and more pinnately dissected. Fertile fronds are more finely dissected and their enrolled margins are covering the sporangia.

**Developmental and tissue-specific variation in Ceratopteris richardii cell walls (right hand side).** Localization of cell wall components in hermaphroditic gametophytes and in transverse sections of sporophytic petioles. Calcofluor white stains β-glucans such as cellulose, which occurs in most cell walls. A xyloglucan epitope (mAb LM15) is detected in the apical neck cells of fully mature (and opened) archegonia. LM11, a mAb directed against xylan labeled secondary cell walls of the petiole. d, developing archegonium; m, mature and opened archegonium; mAb, monoclonal antibody; s, sclerenchyma; t, tracheids. Scale bars = 20 μm.

“ hallmark” of vascular plants (Wing et al., 2008), are not homologous between the lycophyte and euphyllophyte vascular plant lineages. Lycophytes also have unique primary cell wall characters. The isolation of uniquely high concentrations of the unusual sugar residue 3-O-methyl-D-galactose had previously been considered an autapomorphy of the lycophytes as its occurrence was restricted to homosporous (including Lycopodium pennifolium, Huperzia selago, and Diphasiastrum alpinum) and heterosporous lycophyte (including three species of Selaginella although not S. moellendorfii) primary cell walls (Popper et al., 2001).

Despite accounting for only 5–10% of the dry mass of cell walls (Jann et al., 2008) proteins are intrinsically responsible for wall synthesis, structure and function, primarily through their modification of other cell wall components, such as polysaccharides, in response to developmental and environmental cues. There appears to be a phylogenetic basis to the profile of cell wall-acting enzymes possessed by a specific plant. While some enzyme activities, such as xyloglucan endotransglucosylase, which coordinates expansive plant cell growth by cutting and rejoining of intermicrofibrillar xyloglucan chains (Fry et al., 1992; Nishitani and Tominaga, 1992), appear to be present in all vascular plants (Vissenberg et al., 2003) others show a disjunctive between lycophytes and euphyllophytes. Franková and Fry (2011) extracted and assayed proteins from 57 rapidly growing plant organs from a range of flowering plants, Selaginella (apoda), a horsetail and a liverwort and found remarkable differences in glycanase (endo-hydrolase) and glycosidase (exo-hydrolase) activities which correlated with differences in wall composition. For instance, β-mannosidase activities were highest in plants with mannan-rich endosperms requiring rapid metabolism during germination rather than in plants, including Selaginella, whose vegetative tissues have mannan-rich cell walls (Franková and Fry, 2011). Polygalacturonases (PGs) are a large family of hydrolytic enzymes (Kim et al., 2006) which modify pectins developmentally. Analysis of Arabidopsis, Populus, rice, Selaginella, and Physcomitrella genomes indicate an expansion of the PG gene family occurred after the divergence of the lycophytes and euphyllophytes; 16 PG genes were identified in the lycophtye Selaginella in comparison with 44 in rice and 75 in Populus (Yang et al., 2013). Although lycophytes and euphyllophytes have shared characteristics including vascular tissue and a dominant...
sporophyte generation they last shared a common ancestor 400 million years ago (Pryer et al., 2004) and there are many differences as summarized in Table 1. A fundamental difference between the groups is that lycophytes possess microphylls whereas euphyllophytes possess structurally more complex, particularly with respect to vascularisation, megaphyll leaves (Cronk, 2009). The two organs appear to be developmentally and morphologically distinct which, in combination with the existence of many leafless but otherwise highly complex fossils, has led to relative consensus that despite having similar functional roles microphylls and megaphylls are not homologous (Cronk, 2009). Yang et al. (2013) hypothesized that expansion of the PG gene family may be correlated with the evolution of leaves and increased organ complexity but emphasized that the current sample of sequenced vascular plant genomes, which does not yet include ferns, does not enable dating of the PG gene family expansion. However, spatial-temporal changes in remodeling of cell wall components, such as pectins by PGs, lead to changes in wall biomechanical properties, resulting in altered development and morphology (Boudaoud, 2010).

As outlined above the distinct differences in cell wall biochemistry between lycophytes and euphyllophytes is perhaps not unexpected because lycophytes are distinguished as a sister group to all other vascular plants with associated key differences in anatomy and development (Judd et al., 1999; Pryer et al., 2001; Banks, 2009). Therefore, a model fern may provide key insight into whole plant development (Tilney et al., 1990; Racusen, 2002) and the impact of cell wall metabolism.

C-Fern CELL WALLS

A strong foundation for using *Ceratopteris richardii*, often referred to as C-Fern, as a model to investigate the influence of cell walls on development has been laid by anatomical and cytological investigations. Such studies include scanning electron microscopy of xylem (Carolquist and Schnecker, 2000), gametophyte development (Banks, 1999), embryogenesis (Johnson and Renzaglia, 2008), the histology of spermatocyte cell wall composition (Cave and Bell, 1973) and drug-induced perturbation of cellulose synthesis in root hairs (Meekes, 1986). The latter study indicated that C-Fern responds to cell wall-acting drugs in a similar way to flowering plants. Additionally C-Fern is highly sensitive and provides opportunity to investigate drug action; in a single cell layer, in free-living haploid tissues (gametophytes), and in combination with microtubule organizing centers which might be important in order to investigate the effects of microtubule disruption on cell wall component secretion (Meekes, 1986). Furthermore, an array of C-Fern mutants exists including some that may have altered cell walls. One of the most striking is polka dot, which has clumped chloroplasts, putatively resulting from disruption to the cytoskeleton (Vaughn et al., 1990), which may have led to the observed associated weaknesses in spore walls.

### Table 1 | Summary of differences between the lycophyte Selaginella moellendorffii, fern *Ceratopteris richardii*, and angiosperms

<table>
<thead>
<tr>
<th>Character</th>
<th>Selaginella moellendorffii</th>
<th>Ceratopteris richardii</th>
<th>Flowering plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxonomic grouping</td>
<td>Lycophyte</td>
<td>Fern</td>
<td>Angiosperms</td>
</tr>
<tr>
<td>Ploidy of sporophytes</td>
<td>Diploid</td>
<td>Diploid</td>
<td>Various</td>
</tr>
<tr>
<td>Dominant generation</td>
<td>Sporophyte</td>
<td>Sporophyte</td>
<td>Sporophyte</td>
</tr>
<tr>
<td>Gametophytes</td>
<td>Endosporic (remain largely enclosed in spore tissue), subterranean</td>
<td>Exosporic and photosynthetic</td>
<td>Endosporic (remain enclosed in spore tissue)</td>
</tr>
<tr>
<td>Primary photosynthetic organ</td>
<td>Microphylls, typically with only a single unbranched vascular strand</td>
<td>Megaphylls (euphylls), lateral organs of the shoot, derived from stems and possessing branched vasculature</td>
<td>Megaphylls (euphylls)</td>
</tr>
<tr>
<td>Plant axis</td>
<td>Rhizophore, homorhizic roots (which develop laterally relative to the embryonic axis of the embryo), and stem</td>
<td>Homorhizic roots, and stem</td>
<td>Allorhizic roots (which develop at the opposite end of the embryonic axis to the shoots (eudicots), or a secondarily homorhizic root system (most monocotyledonous plants), and stem</td>
</tr>
<tr>
<td>Mega- and micro-sporangia</td>
<td>Heterosporous, typically producing four megaspores in the megasporangium and hundreds of micro-spores in the micro-sporangium</td>
<td>Homosporous, producing hermaphroditic and male gametophytes</td>
<td>Heterosporous, producing a dispersed ovule (megasporangium protected by an integument)</td>
</tr>
<tr>
<td>Branching pattern</td>
<td>Dichotomous (derived from dichotomous branching of the shoot apical meristems)</td>
<td>Lateral</td>
<td>Lateral</td>
</tr>
</tbody>
</table>
C-Fern AS A MODEL PLANT

Clearly, as previously voiced by others (Weng et al., 2008; Cronk, 2009), there is a requirement for sequenced fern genomes. Although there are currently no fully sequenced fern genomes the National Center for Biotechnology Information’s (NCBI) short read archive (SRA) database has incomplete genome data for two ferns, Ceratopteris richardii and, the perhaps more universally familiar invasive, Pteridium aquilinum (http://www.ncbi.nlm.nih.gov/). The Pteridium sequence is derived from a gametophyte transcriptome (Der et al. 2011) similarly the C-Fern expressed sequenced tags (ESTs) are from the early stages of development in germinating spores (Salmi et al., 2005); both sequences are therefore equivalent to the tissues which give rise to pollen grains and embryos sac in flowering plants. Clearly although wall synthesis and restructuring are required for gametophyte development, particularly cell division and expansive cell growth, less than 1% of the gene products expressed in Ceratopteris spores are cell wall-localized (Salmi et al., 2003). Since annotation was carried out by BLAST comparison with the Arabidopsis genome one possibility is that fern and flowering plant cell wall-localized genes are significantly divergent.

Leptosporangiate ferns, of which Ceratopteris richardii and Pteridium aquilinum are members, comprise over 95% of extant fern diversity (Schuettpelz and Pryer, 2008). In fact both of the aforementioned species belong to the ploypods, a clade strongly supported by molecular and morphological characters including sporangia which possess a vertical annulus interrupted by the stalk (Pryer et al., 2001; Schuettpelz and Pryer, 2008). However, whereas Pteridium is placed in the small dennstaedtioid clade, Ceratopteris belongs to the large, diverse pteridoid clade which accounts for about 10% of extant fern diversity (Schnedler 2004; Schuettpelz and Pryer, 2008), this suggests that Ceratopteris is likely to be highly representative of other ferns. Ceratopteris is homosporous and produces hermaphroditic and male gametophytes (see Figure 1). The male gametophytes are produced in response to antheridiogen (Schiedbauer and Klekowski, 1972). The diploid sporophytes are extremely heteroblastic, initially producing entire sterile leaves and progressing to highly dissected fertile leaves which, under culture conditions, produce many spores continuously throughout the year with sporangia on their crenulated leaf margins (Hickok et al., 1987; Figure 1, left hand side). In comparison to many other ferns including Pteridium, Ceratopteris sporophytes are relatively small, reaching 30–40 cm in height. This feature particularly coupled with its ease of growth in culture has been responsible for the widespread application of Ceratopteris as model in undergraduate plant biology teaching, for example to demonstrate plant life cycles, genetics and development, and in research laboratories (Hickok et al., 1987, 1998; Calie, 2005; Spira and Knisely, 2008). This has led to the development of specific tools and techniques including mutant generation, selection and characterization; mutants include abscisic acid (Hickok, 1985), herbicide-tolerant (Hickok and Schwarz, 1986) and salt-tolerant (Warne et al., 1995). Other features which make Ceratopteris a suitable model include: (1) a short sexual life cycle which can be completed in under 120 days, (2) continuous and abundant spore production, (3) spores that can be stored and remain viable for many years, (4) gametophytes which can be self-fertilized to generate completely homozygous sporophytes, (5) visible microtubule organizing centers and developmental synchrony of cells within a single gametophyte (Hoffman and Vaughan, 1995), (6) sporophytes that can be vegetatively propagated from marginal leaf buds or gemmae allowing maintenance of even sterile mutants (Hickok et al., 1987) and (7) amenability to mutagenesis. Furthermore, although experiments initially suggested that Ceratopteris is resistant to Agrobacterium-mediated transformation (Hickok et al., 1987) Agrobacterium has now been shown capable of transforming Ceratopteris halichrooides (and Chinese brake fern, Pteris vittata) spores leading to stably transformed plants; inheritance analyses revealed stable expression of the transgene in second generation sporophytes (Muthukumar et al., 2013). Additionally, Ceratopteris gametophytes have been shown to take up DNA and RNA directly enabling elucidation of gene function through observation of phenotype following targeted silencing (Stoot et al., 2005; Kusai-Iyosuka et al., 2004; Rutherford et al., 2004).

LOCATION, LOCATION, LOCATION AND FUTURE PERSPECTIVE

Although a fully sequenced fern genome will be available in the near future, likely contributing much to our understanding of the evolution of euphyllophytes, plant cell wall components and their biosynthesis, it is unlikely to reveal the full story. The reason for this is that many wall components are deposited in a tissue, cellular or even sub-cellular fashion, often in response to development (Leroux et al., 2007, 2011). Therefore, genomic studies will yield most information when carried out in combination with localization of wall components using (immuno)cytochemical methods (Cave and Bell, 1973; Hervé et al., 2011). Many of the mAbs and CBMs developed to flowering plant cell walls have the ability to recognize and bind to epitopes present in bryophyte (Ceratopteris et al., 2005) and fern (Leroux et al., 2007, 2011) cell walls including those of C-Fern (as exemplified by Figure 1). The ability to apply these techniques to Ceratopteris (and other ferns) provides advantages for investigating plant development involving the cell wall, not afforded by earlier diverging vascular plants. For example Selaginella gametophytes are endosporic, meaning that the female gametophyte remains ensheathed in spore tissue and subterranean. Flowering plant gametophytes are similarly embedded in sporophyte tissues. In contrast fern gametophytes which are photosynthetic and free-living can be prepared (relatively) easily for biochemical analysis. Furthermore, it is possible to follow every cell throughout development. A fern model, such as Ceratopteris, once sequenced will build on what has already been uncovered by investigation of other sequenced plants, particularly other vascular plants such as Selaginella, and likely divulge many secrets relating to euphyllophyte cell wall biochemistry, evolution and function.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.