**Abstract**

Various cell types can *trans*-differentiate to a transfer cell (TC) morphology characterized by deposition of polarized ingrowth walls comprised of a uniform layer on which wall ingrowths (WIs) develop. WIs form scaffolds supporting amplified plasma membrane areas enriched in transporters conferring a cellular capacity for high rates of nutrient exchange across apo- and symplasmic interfaces. The hypothesis that reactive oxygen species (ROS) are a component of the regulatory pathway inducing ingrowth wall formation was tested using *Vicia faba* cotyledons. *Vicia faba* cotyledons offer a robust experimental model to examine TC induction as, on being placed into culture, their adaxial epidermal cells rapidly (hours) form ingrowth walls on their outer pericinal walls. These are readily visualized by electron microscopy, and epidermal peels of their *trans*-differentiating cells allow measures of cell-specific gene expression. Ingrowth wall formation responded inversely to pharmacological manipulation of ROS levels, indicating that a flavin-containing enzyme (NADPH oxidase) and superoxide dismutase cooperatively generate a regulatory H₂O₂ signature. Extracellular H₂O₂ fluxes peaked prior to the appearance of WIs and were followed by a slower rise in H₂O₂ flux that occurred concomitantly, and co-localized, with ingrowth wall formation. De-localizing the H₂O₂ signature caused a corresponding de-localization of cell wall deposition. Temporal and epidermal cell-specific expression profiles of *VfrbohA* and *VfrbohC* coincided with those of extracellular H₂O₂ production and were regulated by cross-talk with ethylene. It is concluded that H₂O₂ functions, downstream of ethylene, to activate cell wall biosynthesis and direct polarized deposition of a uniform wall on which WIs form.

**Key words:** Reactive oxygen species, transfer cell, *trans*-differentiation, *Vicia faba*, wall ingrowth.

**Introduction**

Transfer cells (TCs) are specialized plant cells with intricately invaginated wall ingrowths (WIs) ensheathed by an amplified plasma membrane, enriched in a suite of nutrient transporters (Offler et al., 2003; McCurdy et al., 2008). Specialized for membrane transport, TCs occur at sites of intense nutrient exchange across apo-/symplasmic boundaries (Offler et al., 2003). TCs contribute to distributing nutrients throughout the plant body by facilitating nutrient loading (e.g. Amiard et al., 2007; Maeda et al., 2008) and unloading of vascular pipelines into heterotrophic sinks including agronomically important seeds (Zhang et al., 2007). In other physiological contexts, TCs may augment nutrient flows between plant host and biotroph (symbiont/pathogen; Offler et al., 2003) as well as play important roles in adaptive responses to combat soil nutrient deficiencies (e.g. Schikora and Schmidt, 2002) and salt stress (e.g. Boughanmi et al., 2003). At these tissue sites of intense nutrient exchange, TCs form exclusively by *trans*-differentiating from a range of...
vascular and non-vascular cell types (Offler et al., 2003). Despite TCs fulfilling key physiological roles in nutrient transport and plant productivity, the signal(s) and signalling cascades responsible for initiating their trans-differentiation are poorly understood.

Thus far exploration of induction of TC trans-differentiation has identified auxin (Dibley et al., 2009), ethylene (Zhou et al., 2010), and glucose (Andriunas et al., 2011) as central regulators of WI induction. TC induction undoubtedly requires coordinate interaction between multiple signal transduction pathways to mediate the rapid transcriptional changes accompanying the trans-differentiation process (Dibley et al., 2009). Ethylene and glucose signalling pathways are known to interact with other signals including reactive oxygen species (ROS; Bolouri-Moghaddam et al., 2010).

ROS encompass a variety of reactive oxidants including singlet oxygen, superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH$^-$. The enzymatic source of the highly reactive O$_2^-$ is one or more NADPH oxidase (NOX) isoforms (Van Breusegem et al., 2008). In plants, NOX consists of a plasma membrane-bound NADPH-binding flavocytochrome that forms an electron transport chain capable of reducing oxygen (O$_2$) to O$_2^-$ (Sagi and Fluhr, 2006). Genes encoding plant NOX are called respiratory burst oxidase homologues (rboh) genes since they share homology to gp91$^{phox}$, the catalytic subunit of mammalian NOX/respiratory burst oxidase (Sagi and Fluhr, 2006). The highly reactive O$_2^-$ rapidly (milliseconds) undergoes dismutation to form H$_2$O$_2$, either spontaneously or via the superoxide dismutase (SOD) enzyme, which further accelerates the reaction by $\sim$400-fold (Mori and Schroeder, 2004). Although H$_2$O$_2$ is less reactive than O$_2^-$, it is more stable, water-soluble, and can diffuse through membranes via aquaporins (Bienert et al., 2007). H$_2$O$_2$ is therefore recognized as the most potent signalling ROS in plants (Van Breusegem et al., 2008).

ROS have emerged as important regulators of developmental processes in plants (Van Breusegem et al., 2008), including cell wall construction that resonates with trans-differentiation to a TC morphology (McCurdy et al., 2008). For instance, ROS function as developmental signals in the differentiation of the cellulose-rich cell wall in cotton fibres (Potikha et al., 1999). Thus, ROS are likely candidate molecules to regulate WI formation in TCs. This supposition is strengthened by the observation that WI development is linked in many instances to stress responses (see Offler et al., 2003). For example, WI deposition in leaf phloem parenchyma cells is enhanced following exposure to high light and cold (Amiard et al., 2007; Maeda et al., 2008; Edwards et al., 2010), while root rhizodermal cells trans-differentiate to a TC morphology as an adaptive response to nutrient deficiency (Schikora and Schmidt, 2002) and salinity (Boughnami et al., 2003). ROS production is a common response to these stresses (Miller et al., 2010) and therefore might be linked to TC induction. For example, suppressed WI formation in phloem parenchyma cells of Arabidopsis GIGANTEA mutants (Edwards et al., 2010) might be due to their enhanced ability to detoxify/scavenge ROS (Cao et al., 2006), potentially reducing ROS levels. Conversely, enhanced deposition of WIs in phloem parenchyma TCs in an Arabidopsis vitamin E (vte) mutant might be due to the deficiency in production of tocopherol, a key ROS scavenger (Maeda et al., 2008), potentially increasing ROS levels.

However, there is no direct evidence that ROS are part of a regulatory pathway that induces cells to undergo a set of trans-differentiation events leading to construction of a TC wall morphology comprised of a uniform wall layer on which WIs form (McCurdy et al., 2008). This composite wall structure is defined as an ingrowth wall. In this context, a series of experimental observations were undertaken to test whether ROS regulated any component of ingrowth wall construction by utilizing adaxial epidermal cells of cultured Vicia faba cotyledons. Upon placing excised cotyledons into culture, their adaxial epidermal cells are rapidly (hours) induced to trans-differentiate to form a polarized ingrowth wall identical to the one formed in planta by their abaxial counterparts (Wardini et al., 2007, and references therein). Such a plant model provides experimental access to a large population of trans-differentiating epidermal cells combined with an ability to visualize developing WI papillae with ease (see Zhou et al., 2010). The presence of WI papillae signals that an ingrowth wall has been formed. Thus impacts of pharmacologically altered ROS levels on induction of ingrowth wall formation were determined using the presence/absence of WI papillae coupled with localizing ROS production at the cell level. In addition, the role of ROS in directing polarized deposition of the uniform wall, that precedes WI formation, was evaluated. Three NOX genes were cloned and their temporal and spatial expression evaluated during TC induction. Potential interactions between ROS and ethylene were examined at the cell and molecular levels to build an integrated understanding of the regulatory pathway responsible for ingrowth wall induction.

Materials and methods

Plant material and growth conditions

Vicia faba L. cv. Fiord plants were grown according to Zhou et al. (2010).

Cotyledon culture

Vicia faba cotyledons were isolated and prepared for aseptic culture on a Murashige and Skoog (1962) medium (MS) following a protocol outlined by Zhou et al. (2010). Briefly, sister cotyledon pairs were divided between MS medium with and without specified additions. For diphenyleneiodonium (DPI) treatment, cotyledons were first pre-treated on ice for 1 h in Petri dishes containing 300 mM betaine ± DPI to load the cells with the inhibitor while metabolism was attenuated. Cultures were then placed in darkness at 26 $^\circ$C for 15 h unless specified otherwise.

Electron microscopy and estimates of cell wall widths

Tissue was prepared for scanning electron microscopy (SEM) as described by Zhou et al. (2010). For transmission electron microscope (TEM) observations, 2 mm$^2$ pieces of cultured cotyledons were fixed and embedded in London Resin (LR) White resin (for details, see Offler et al., 1997). Ultrathin (60 nm thick)
transverse sections were stained with saturated uranyl acetate and counterstained with saturated lead citrate, prior to viewing with a JEOl 1200 EX II electron microscope. Averaged estimates of the widths of outer and inner periclinal, as well as anticlinal, walls of adaxial epidermal cells were obtained from TEM images using Image J software (http://rsbweb.nih.gov/ij/) to measure surface areas occupied by each wall face expressed on a length basis (i.e. \( \text{nm}^2 \text{nm}^{-1}\text{nm} \)). Twelve to 15 epidermal cells were measured per replicate and there were four replicate cotyledons per treatment.

Since WIs represent the final layer of an ingrowth wall (McCurdy et al., 2008) and the SEM procedure used to visualize these structures (see above) allows a high throughput of tissue samples to yield statistically robust data sets, percentages of adaxial epidermal cells containing WIs were routinely used as a measure of ingrowth wall formation. However, where necessary, SEM observations were supplemented with TEM observations of transverse sections of adaxial epidermal cells to visualize impacts of various treatments on uniform wall layer formation.

Measurement of extracellular \( \text{H}_2\text{O}_2 \)

The Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazone; Invitrogen, Australia) was utilized to measure extracellular \( \text{H}_2\text{O}_2 \) release from cotyledons, in a procedure adapted from Ortega-Villasante et al. (2007). Cotyledons were cultured on MS medium ± ROS inhibitors and scavengers for specified culture periods. Thereafter cultured cotyledons were rinsed in distilled water (3×1 min) to remove apoplasmic solutes. Following rinsing, cotyledons were placed with their adaxial surfaces down in individual wells of a 24-well culture plate. Each well was filled with 200 \( \mu \text{l} \) of 25 mM phosphate-buffered saline (pH 7) containing 50 \( \mu \text{M} \) Amplex Red, horseradish peroxidase (0.1 U ml\(^{-1}\)), and the pharmacological compound on which cotyledons had been cultured. Well plates were covered with foil and placed at 26 °C for 15 min. The solution was immediately aliquoted into microcuvettes and absorbance measured at 560 nm. Bath concentrations of \( \text{H}_2\text{O}_2 \) were determined from a standard curve prepared in each solution described above. Net fluxes of \( \text{H}_2\text{O}_2 \) release into the bath solution (nmol min\(^{-1}\)) were calculated for each culture time point. Cotyledons were image scanned, and Image J software (http://rsbweb.nih.gov/ij/) was utilized to determine their adaxial surface area exposed to the Amplex Red solution. Net fluxes of \( \text{H}_2\text{O}_2 \) release were then expressed per mm\(^2\) of the adaxial cotyledon surface. The short exposure time to the bath solution (15 min) combined with \( \text{H}_2\text{O}_2 \) escaping to the larger volume of bath solution where \( \text{H}_2\text{O}_2 \) was diluted to nanomolar concentrations are considered collectively to minimize, but not exclude, loss of \( \text{H}_2\text{O}_2 \) through metabolism escaping to the larger volume of bath solution where \( \text{H}_2\text{O}_2 \) was diluted to nanomolar concentrations are considered collectively to minimize, but not exclude, loss of \( \text{H}_2\text{O}_2 \) through metabolism of \( \text{H}_2\text{O}_2 \). Histochemical detection of \( \text{H}_2\text{O}_2 \) using diaminobenzidine

To localize \( \text{H}_2\text{O}_2 \) production histochemically, 3′,3′-diaminobenzidine (DAB; Sigma, Australia) was utilized in a procedure adapted from Thordal-Christensen et al. (1997). Cotyledons were cultured, adaxial surface down, in Petri dishes containing 10 ml of liquid MS/DAB solution \( \pm 100 \mu \text{M} \) DPI or \( \pm 10 \mu \text{M} \) \( \text{H}_2\text{O}_2 \). Following culture, selected cotyledons were prepared for SEM and scored for WI formation to verify that incubation in DAB did not affect the extent of WI induction or the response to DPI (Supplementary Fig. S1 available at JXB online). The remaining cotyledons (six per treatment) were fixed in 3% (v/v) glutaraldehyde, 4% (v/v) paraformaldehyde in 10 mM sucrose buffered to pH 7 with 50 mM piperazine-N,N′-bis 2-ethanesulphonic acid (PIPES). Fixation was carried out for 4 h at 4 °C, with the fixative changed after 1 h and 2 h. Following a buffer wash, fixed cotyledons were dehydrated through a 10% step-graded ethanol dilution series and ultimately infiltrated and embedded in LR White resin (for more details, see Offler et al., 1997). Embedded cotyledons were sectioned transversely at 1 μm thickness. Sections were viewed by bright-field microscopy using a Zeiss Axiophot microscope and images recorded with a Zeiss AxioCam HCr camera using Axiovision software.

Cloning \( \text{rboh} \) genes

Total RNA was extracted from adaxial epidermal cells of \textit{V. faba} cotyledons, and cDNA was synthesized as described by Zhou et al. (2010). The resulting cDNA was subjected to degenerate PCR using the primers listed in Supplementary Table S1 at JXB online. Partial gene fragments were cloned according to methods outlined by Zhou et al. (2010). At least 10 clones were sequenced for each gene fragment. Full-length \( \text{rboh} \) genes were isolated by 5′ and 3′ SMART RACE RT-PCR (Clontech). Sequences were analysed using Sequencher (version 4.1; Gene Codes Corporation, Ann Arbor, MI, USA). The full-length sequences for \( \text{VfrbohA} \) and \( \text{VfrbohC} \) and the partial sequence for \( \text{VfrbohB} \) were deposited in GenBank under the accession numbers JF784279, JF784280, and JF784281, respectively. Predicted amino acid sequences of cloned \( \text{rboh} \) genes were compared for identity and aligned using ClustalX2 (Larkin et al., 2007).

Quantitative real-time PCR

Real-time PCR was conducted on cDNA derived from adaxial epidermal and storage parenchyma tissue of uncultured and cultured cotyledons as described by Zhou et al. (2010). Transcript levels were normalized to \( \text{V. faba elongation factor} \) \( \alpha \) (VfEF\( \alpha \)). To determine comparable transcript levels between the \( \text{rboh} \) isoforms, an equivalent amount of RNA was used for cDNA synthesis in all replicates and time points, and absolute transcript levels of all genes were determined from equivalent cDNA samples within the same real-time PCR run. Standard curve thresholds were set to equivalent values for all genes, in order to quantify accurately and compare directly transcript levels between the \( \text{rboh} \) isoforms utilizing the Corbett Rotor-Gene 6000 software package (version 1.7).

Statistical analyses

Statistical significance of treatment effects on WI induction, gene expression, or rates of ROS release were determined using the Student’s \( t \)-test.

Results

Manipulation of ROS levels impacts wall ingrowth induction

To test the role of ROS in regulating induction of TC transdifferentiation in adaxial epidermal cells, ROS levels were manipulated across 15 h culture of \textit{V. faba} cotyledons utilizing ROS biosynthesis inhibitors and scavengers, and their impact on WI formation was determined.

Culture of cotyledons on a medium containing DPI, a potent inhibitor of NOX and other flavin-containing enzymes (Gapper and Dolan, 2006), suppressed induction of adaxial epidermal cells to form WIs by 9-fold (Fig. 1A). For the 10% of DPI-treated cells in which WIs were induced (Fig. 1A), morphologies of WI papillae were identical to those in control cotyledons; however, their densities were lower (Supplementary Fig. S2 at JXB online). DPI-mediated suppression of WI induction was reversed completely by removing cotyledons from the DPI-containing medium and

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culturing for an additional 15 h on a DPI-free medium (Fig. 1A). This recovery discounted possible non-specific effects resulting from toxicity of the inhibitor. A comparable response was obtained by culturing cotyledons in the presence of DPI and H₂O₂ (Fig. 1A). Consistent with WI formation being sustained by exogenous H₂O₂ when ROS production was blocked by DPI, papillae morphology and densities of DPI+H₂O₂-treated cells were identical to those of control cotyledons (Supplementary Fig. S2).

Reduction of O₂ to O₂/C₀ is catalysed by NOX (Sagi and Fluhr, 2006). H₂O₂ is subsequently formed spontaneously or by SOD-mediated dismutation of O₂ (Mori and Schroeder, 2004). To evaluate any requirement for SOD activity, cotyledons were cultured for 15 h on a medium containing diethyldithiocarbamate (DDC), an inhibitor of Cu/Zn-SOD (Delledonne et al., 2001). Under these conditions, WI induction was suppressed 4-fold (Fig. 1B), providing evidence that the phenomenon was SOD dependent and the regulatory ROS species was H₂O₂ (Fig. 1A).

To verify further that the above conclusions did not result from ambiguities introduced by the pharmacological agents used, cotyledons were cultured on a panel of known H₂O₂ scavengers, including catalase, ascorbic acid (AA), and reduced glutathione (GSH; Foyer and Noctor, 2005). These treatments resulted in a 7-, 5-, and 4-fold suppression of WI induction, respectively (Fig. 1A), consistent with H₂O₂ exerting a key regulatory influence over WI and hence ingrowth wall formation.

Two sequential bursts of extracellular ROS production with the first preceding, and the second accompanying, WI induction

To determine if extracellular ROS production correlated with WI induction, and to verify sensitivity to ROS

![Graph A](image1)

**Fig. 1.** Effect of diphenyleneiodonium (DPI), hydrogen peroxide (H₂O₂), diethyldithiocarbamate (DDC), and ROS scavengers on induction of WIs in adaxial epidermal cells of *Vicia faba* cotyledons. (A) Percentages of adaxial epidermal cells containing WIs in cotyledons cultured for 15 h on MS medium (control), MS medium containing +100 μM DPI, or MS medium containing both 100 μM DPI and 10 μM H₂O₂. In one treatment, following 15 h culture on MS medium containing 100 μM DPI, cotyledons were subsequently transferred to DPI-free MS medium for a further 15 h. Data represent the mean ± SE for six replicate cotyledons (*P < 0.0001 for each treatment against control). (B) Percentages of adaxial epidermal cells containing WIs in cotyledons cultured for 15 h on MS medium (control), or MS medium containing either 1 mM DDC, catalase (1000 U), 10 mM ascorbic acid (AA), or 10 mM reduced glutathione (GSH). Data represent the mean ± SE for six replicate cotyledons (*P < 0.0001 for each treatment against control).
Rboh genes are up-regulated specifically in adaxial epidermal cells prior to, and during, wall ingrowth induction

Rboh genes encode the catalytic subunit of NOX in plants (Sagi and Fluhr, 2006). To gain insight into the rboh isoform(s) responsible for generating apoplastic ROS (Fig. 2), rboh genes were cloned by degenerate PCR from adaxial epidermal cells of cultured cotyledons, and their temporal and spatial expression patterns determined.

Three rboh genes were identified from sequencing 40 degenerate PCR clones, of which two full-length (VfrbohA and VfrbohC) and one partial (VfrbohB) cDNA sequence were obtained (Supplementary Fig. S4 at JXB online). Comparison of partial predicted amino acid sequences of VfrbohA and VfrbohC, corresponding to VfrbohB, demonstrated that VfrbohA and VfrbohB were most closely related, sharing 84% identity (Supplementary Fig. S4). In contrast, VfrbohC shared 54% identity with VfrbohB (Supplementary Fig. S4). The full-length predicted amino acid sequences of VfrbohA and VfrbohC shared 65% identity (Supplementary Fig. S4). All three sequences contained motifs common to human NOX protein gp91phox (Sagi and Fluhr, 2006) and potential flavin adenine dinucleotide (FAD)- and NADPH-binding domains in the C-terminal regions (Segal et al., 1992; and see Supplementary Fig. S4). Full-length VfrbohA and VfrbohC contained characteristic features of plant rbohs including two Ca$^{2+}$-binding EF hands in their N-terminal regions (Keller et al., 1998; and see Supplementary Fig. S4). Consistent with Ca$^{2+}$ positively regulating the activities of rbohs by binding to their EF hands (Kudla et al., 2010), depletion of extracellular Ca$^{2+}$ by the chelator 1,2-bis(o-aminophenoxy)ethane-N, N, N, N-tetraacetic acid (BAPTA) substantially reduced net fluxes of H$_2$O$_2$ released from cotyledons, with the greatest impact (3-fold) evident during the first H$_2$O$_2$ burst (Fig. 2). Based on these sequence characteristics and the response of net extracellular H$_2$O$_2$ production to an altered Ca$^{2+}$ supply, it is concluded that the three rboh genes, expressed in adaxial epidermal cells of cultured V. faba cotyledons, encode the key enzymatic subunit of the plant NOX.

Phylogenetic analysis (Supplementary Fig. S5 at JXB online) demonstrated that VfrbohA was closely related to Mtrboh2 that functions to maintain the polarity of root hair development (Lohar et al., 2007). The analysis was less informative for VfrbohC that clustered with a cohort of rbohs known to contribute to extracellular ROS production. For instance, NtrbohF and NbrbohA are responsible for ROS accumulation during plant defence and hypersensitive responses (Torres et al., 2002; Yoshioka et al., 2003).

Temporal and spatial expression profiles of cloned rboh genes were derived from real-time PCR of cDNA obtained from adaxial epidermal peels and storage parenchyma discs harvested at various times across cotyledon culture (Dibley et al., 1998; and see Supplementary Fig. S4). Fig. 3. Expression analyses of respiratory burst oxidase homologue (rboh) genes in cultured Vicia faba cotyledons. Relative temporal and spatial expression profiles of VfrbohA (A), VfrbohB (B), and VfrbohC (C) in adaxial epidermal (ep) and storage parenchyma (sp) tissues of cotyledons during 15 h culture. The relative expression of each gene determined by real-time PCR was normalized to expression of Vicia faba elongation factor $\alpha$. Data represent the mean ± SE for four replicate cotyledons. The arrow indicates the first appearance of WIs (Wardini et al., 2007).
et al., 2009). Up to 3 h of cotyledon culture, the temporal expression profile of VfrbohA correlated with the pattern of net extracellular ROS production (compare Fig. 3A with Fig. 2). Expression of VfrbohA peaked rapidly and specifically in adaxial epidermal cells prior to WI induction (Wardini et al., 2007), with relative transcript levels at 0.5 h >200-fold higher than those of uncultured cotyledons (Fig. 3A). Expression thereafter declined and remained low between 6 h and 15 h of cotyledon culture (Fig. 3A). In contrast, expression of VfrbohA in storage parenchyma tissues remained extremely low and stable throughout the entire culture period.

VfrbohB expression was also up-regulated specifically in adaxial epidermal cells, but temporally lagged behind that of VfrbohA and extracellular ROS production (compare Fig. 3B with Fig. 2). Prior to WI induction, up-regulated expression of VfrbohC was moderate compared with that of VfrbohA and VfrbohB. However, in contrast to its two counterparts, following a slight decline between 1 h and 3 h, expression of VfrbohC increased to its peak level observed prior to WI induction, and thereafter remained stable during 6–15 h culture (Fig. 3C). Expression of VfrbohC in storage parenchyma followed a similar temporal profile to adaxial epidermal cells, but was 3-fold lower prior to WI induction and >6-fold lower during the WI induction period (3–15 h; Fig. 3C).

To gain further insight into the chief rboh isoform(s) that may contribute to the peak in ROS production prior to WI induction, transcript levels were compared directly between the three rboh isoforms in adaxial epidermal cells. Prior to culture, there was little difference in expression between VfrbohA, VfrbohB, and VfrbohC, with all genes expressed at very low levels (Table 1). Following 1 h of culture, VfrbohA was the most highly expressed gene, with transcript levels >1000-fold higher than those of VfrbohB and 22-fold greater than those VfrbohC (Table 1). Cell-specific expression of the three Vfrboh genes (Fig. 3) falls within the range of rboh numbers expressed in particular cell types reported for Arabidopsis (Suzuki et al., 2011). This observation, combined with the three Vfrboh genes being identified after sequencing 40 degenerate PCR clones, provides strong circumstantial evidence that VfrbohA, VfrbohB, and VfrbohC are likely to be the major rboh genes expressed in adaxial epidermal cells undergoing trans-differentiation to a TC morphology.

Overall, temporal and spatial expression profiles (Fig. 3), along with transcript levels (Table 1), suggest that VfrbohA may be responsible for rate-limiting ROS production prior to WI induction. Thereafter, during the phase of WI induction, VfrbohC could assume a greater role in ROS production (Fig. 3C). In all cases, each rboh isoform was expressed selectively in adaxial epidermal cells undergoing trans-differentiation to a TC morphology (Fig. 3).

### Table 1. Comparison of transcript levels of respiratory burst oxidase homologue (rboh) genes in adaxial epidermal cells of Vicia faba cotyledons

| Culture period (h) | Absolute transcript level (10^−4) | VfrbohA | VfrbohB | VfrbohC |
|-------------------|----------------------------------|---------|---------|---------|
| 0                 |                                  | 0.11±0.04 | 0.00±0.00 | 0.30±0.11 |
| 1                 |                                  | 18.03±1.82 | 0.02±0.00 | 0.83±0.17 |

ROS contribute to polarizing ingrowth wall formation

To localize cellular sites of H2O2 production during WI induction, cultured cotyledons were exposed to DAB and

![Fig. 4. Localization of hydrogen peroxide (H2O2) in transverse sectioned Vicia faba cotyledons during wall ingrowth induction. Representative light micrographs of transverse sectioned cotyledons following culture for 15 h on MS medium alone (A), or MS medium containing either 100 μM diphenyleneiodonium (DPI; B) or 10 μM hydrogen peroxide (H2O2; C). All media contained 1 mg ml−1 diaminobenzidine (DAB) to localize H2O2 detected as H2O2-induced DAB polymerization visualized as brown precipitates indicated by arrows, noting that (B) exhibits an extremely faint signal. ec, adaxial epidermal cell; sp, storage parenchyma cell. Bar=20 μm.]
transverse sections visualized by light microscopy. DAB polymerizes at sites of H$_2$O$_2$ production to form an insoluble brown precipitate (Thordal-Christensen et al., 1997). The H$_2$O$_2$ signal was polarized to outer periclinal walls of adaxial epidermal cells undergoing WI induction (Fig. 4A), and hence co-localized with polarized deposition of ingrowth walls in these cells (see Offler et al., 1997). The staining intensity was reduced dramatically by exposing cotyledons to DPI (Fig. 4B), suggesting that the DAB-detected ROS signature was generated by NOX activity and rapidly reduced to H$_2$O$_2$ (see also Figs 1 and 2). As a positive control, cotyledons were cultured on 10 $\mu$M H$_2$O$_2$. This treatment resulted in staining

**Fig. 5.** Transmission electron microscope (TEM) images of transverse sections of adaxial epidermal cells of *Vicia faba* cotyledons illustrating the effect of diphenyleneiodonium (DPI) and hydrogen peroxide (H$_2$O$_2$) on ingrowth wall formation. (A–C) Representative TEM images of freshly harvested cotyledons (A), cotyledons cultured for 15 h on MS medium alone (B), or MS medium containing 100 $\mu$M DPI (C). The newly deposited uniform wall is identified with white arrows and wall ingrowths by black arrows with white borders in insets of the outer periclinal wall. Bar=2 $\mu$m for whole-cell images and 1 $\mu$m in insets. (D–I) Representative TEM images of outer periclinal (D, E), anticlinal (F, G), and inner periclinal (H, I) walls of adaxial epidermal cells of cotyledons cultured on MS medium alone (D, F, H) or MS medium containing 10 $\mu$M H$_2$O$_2$ (E, G, I) for 24 h. Wall ingrowths arising from a uniform wall layer are identified by black arrows with white borders. Bar=500 nm.
Table 2. Widths of original outer periclinal wall, newly deposited uniform outer periclinal wall layer, and anticlinal and inner periclinal walls of adaxial epidermal cells of freshly harvested *Vicia faba* cotyledons (t=0) or cotyledons cultured for 15 h or 24 h in the presence or absence of diphenyleneiodonium (DPI) or hydrogen peroxide (H$_2$O$_2$), respectively.

| Treatment       | Wall width (nm) | Original outer periclinal | Deposited uniform outer periclinal | Anticlinal | Inner periclinal |
|-----------------|-----------------|---------------------------|-----------------------------------|------------|-----------------|
| t=0             |                 | 572.4±78.6                | 0                                 | 212.1±12.5 | 202.5±14.4      |
| Control (15 h)  |                 | 632.6±42.1                | 327.2±22.8                        | 231.8±24.3 | 254.9±20.0      |
| 100 µM DPI      |                 | 641.7±24.4                | 89.1±7.6                          | 219.5±6.6  | 260.5±11.5      |
| Control (24 h)  |                 | 602.3±18.7                | 275.6±11.3                        | 180.6±11.2 | 245.1±25.3      |
| 10 µM H$_2$O$_2$|                 | 702.1±22.7                | 404.7±24.0                        | 303.2±15.8 | 302.7±18.2      |

Values are the mean ± SE (n=55).

cell walls across all cotyledon tissues (Fig. 4C), showing that the localized DAB colour reaction in control cotyledons (Fig. 4A) was not the result of localized peroxidase activity (Thordal-Christensen et al., 1997) or an inability of DAB to diffuse throughout the cotyledon apoplast. Moreover, this finding provided the opportunity of experimentally determining the impact of de-localizing the H$_2$O$_2$ signature on ingrowth wall deposition as described below.

Ingrowth walls of TCs are composed of a polarized uniform wall upon which localized papillate projections arise to generate a WI labyrinth (Offler et al., 2003). The question then arises as to whether ROS initiates formation of both the uniform wall and WIs, or either the uniform wall or WIs alone. This question was explored by examining transverse sections of adaxial epidermal cells of freshly harvested (t=0 h) and 15 h cultured cotyledons viewed by TEM and determining their wall widths. Initiating trans-differentiation to a TC morphology upon cotyledon culture induced a uniform wall to be laid down polarized to outer periclinal walls of adaxial epidermal cells and was demarcated from the original wall by an electron-dense band of wall material (Fig. 5B versus A; Table 2). Uniform wall deposition was completed by 15 h of cotyledon culture and was the exclusive site of wall construction within these epidermal cells (Table 2).

Culture of cotyledons on DPI, to dampen ROS production (Figs 2, 4A), significantly inhibited formation of a uniform wall (Fig. 5C versus B; Table 2) and hence WIs (Fig. 1A; Supplementary Fig. S2 at *JXB* online). Moreover, inhibition of uniform wall formation did not result in any deposition being re-directed to other wall interfaces (Table 2). In contrast, de-localizing the apoplastic ROS signature by cotyledon culture on H$_2$O$_2$ (Fig. 4C versus A) caused additional wall material to be deposited on all wall interfaces of adaxial epidermal cells (Fig. 5D–I; Table 2). Nevertheless, a preferential deposition (an ~2-fold increase over control levels) to the outer periclinal wall was evident in the presence of excess H$_2$O$_2$ (Table 2), indicating a continued presence of a polarizing influence on wall construction. Together these observations are consistent with H$_2$O$_2$ functioning to regulate overall activity of wall building machinery as well as exerting a polarizing influence on ingrowth wall formation. Significantly, however, WI formation was restricted to the outer periclinal wall (Fig. 5D versus F–I) irrespective of whether ROS were localized or dispersed around each epidermal cell. This finding suggests that another regulatory molecule, in addition to ROS, is required to induce WI formation.

**Ethylene regulates ROS production**

Auxin (Dibley et al., 2009), ethylene (Zhou et al., 2010), glucose (Andriunas et al., 2011), and ROS (Figs 1–5) regulate induction of ingrowth wall formation in adaxial epidermal cells of *V. faba* cotyledons. Auxin functions upstream of ethylene to drive induction of ethylene biosynthetic genes (Zhou et al., 2010), and ethylene/glucose interact antagonistically to determine flow through an ethylene signal cascade (Andriunas et al., 2011). Possible cross-talk between ethylene and ROS was explored using a pharmacological approach to control biosynthesis/levels of ethylene and ROS differentially.

For cotyledons in which ROS production was suppressed by DPI, augmenting ethylene biosynthetic capacity, by supplying the ethylene precursor aminocyclopropane-carboxylic acid (ACC), did not restore WI formation (Fig. 6A). In contrast, dampening ethylene biosynthetic capacity with an ethylene biosynthesis inhibitor, aminooxycyvinylglycine (AVG), and supplementing with H$_2$O$_2$, resulted in partial recovery of AVG-inhibited WI induction (Fig. 6A). These findings are consistent with H$_2$O$_2$ acting downstream of ethylene to regulate ingrowth wall formation. The proposed ethylene/H$_2$O$_2$ model was substantiated by discovering that net production of extracellular H$_2$O$_2$ was sensitive to modulating ethylene biosynthetic capacity by exposing cotyledons to AVG or ACC (Fig. 6B; and see Zhou et al., 2010).

To determine if ethylene regulates *rboh* expression (Fig. 3), ethylene biosynthesis was manipulated using a 1 h exposure of cotyledons to AVG or ACC, with effects on *rboh* transcript levels in adaxial epidermal cells determined by real-time PCR. Expression of *VfrbohA* was inhibited 7-fold in cotyledons cultured on AVG and enhanced 12-fold when exposed to ACC (Fig. 7A). In contrast, *VfrbohB* and *VfrbohC* expression did not change significantly in response to manipulating ethylene biosynthesis (Fig. 7B and C, respectively). Therefore, ethylene exclusively regulates the peak in *VfrbohA* expression (Fig. 7A) and hence net production of extracellular H$_2$O$_2$ (Fig. 2) prior to WI induction (Wardini et al., 2007). The sensitivity of *VfACS2*
Differentiation

Fluxes of H$_2$O$_2$ released into adaxial epidermal cell walls of Vicia faba cotyledons. Exposure of cultured cotyledons to DPI, an inhibitor of flavin-containing enzymes such as NOX (Gapper and Dolan, 2006), significantly suppressed induction of WIs (Fig. 1A, Supplementary Fig. S2 at JXB online). This finding points to ROS positively regulating TC induction. Significantly, DPI inhibition was abrogated by simultaneous exposure to H$_2$O$_2$ (Fig. 1A; Supplementary Fig. S2), suggesting that H$_2$O$_2$ is the primary ROS molecule regulating TC induction. Consistent with this conclusion, lowering H$_2$O$_2$ levels by a DDC block (Delledonne et al., 2001) of dismutating O$_2$ to H$_2$O$_2$ or by exposure to ROS scavengers was directly linked with these treatments inhibiting WI induction (Fig. 1B). Similar conclusions were drawn for ROS inducing formation of the uniform wall (Fig. 5; Table 2) on which WI papillae form (McCurdy et al., 2008).

Moreover, pharmacological depression of net production of extracellular ROS (Fig. 2) resulted in blocking wall biosynthesis within adaxial epidermal cells during cotyledon culture (Table 2) and hence the cellulose scaffold essential for WI formation (Talbot et al., 2007). Such an action is consistent with H$_2$O$_2$ oxidation mediating CesA homodimerization (Kurek et al., 2002) prior to their oligomerization to form functional CesA rosettes (Atanassov et al., 2009).

Suppressing net production of extracellular H$_2$O$_2$ by DPI (Fig. 2) indicates that the rapid extracellular increase in H$_2$O$_2$ is generated by activities of flavin-containing enzymes such as NOX (Sagi and Fluhr, 2006) but does not exclude contributions by other DPI-sensitive ROS biosynthetic enzymes present in cell walls (e.g. polyamine oxidase; Cona et al., 2001). However, the presence of a H$_2$O$_2$-generating mechanism dependent upon NOX activity is suggested by several independent pieces of circumstantial evidence. For instance, consistent with the strongly expressed Vfrboh-encoded NOX genes (Fig. 3; Table 1) carrying two Ca$^{2+}$-binding EF-hand motifs (Supplementary Fig. S4 at JXB online; Keller et al., 1998), extracellular Ca$^{2+}$ chelation suppressed net production of extracellular H$_2$O$_2$ (Fig. 2; and see Takeda et al., 2008). Moreover, an epidermal cell-specific up-regulation of Vfrboh expression (Fig. 3) coincided with the two consecutive extracellular bursts in net H$_2$O$_2$ production (Fig. 2). The claimed transcriptional control of ROS production by Vfrboh expression is somewhat unusual as to date such a phenomenon appears to be confined to stress responses (e.g. Jakubowicz et al., 2010; Marino et al., 2011; Rajhi et al., 2011).

It is possible a number of rboh isoforms could contribute to the rapid peak in extracellular net production of H$_2$O$_2$ prior to WI induction (Fig. 2). However, several lines of correlative evidence suggest that net extracellular H$_2$O$_2$ production prior to WI induction was rate limited by the VfrbohA-encoded NOX (Supplementary Fig. S4 at JXB online). First, the temporal profile of VfrbohA expression most closely matched that of net ROS production prior to WI induction (Fig. 3A compared with Fig. 2). Secondly, VfrbohA was undoubtly the most abundantly expressed rboh isoform prior to WI induction (Table 1). Thirdly, VfrbohA expression was the only rboh regulated by ethylene (Fig. 7A versus B, C), thereby linking ethylene and ROS exerting regulatory control over expression to DPI and H$_2$O$_2$ (Fig. 7D) suggests feedback regulation of ethylene biosynthesis by ROS.

**Discussion**

*Extracellular ROS production and expression of rboh genes are linked with induction of TC trans-differentiation*

Manipulating ROS levels during cotyledon culture identified ROS as a key player in regulating induction of TC trans-differentiation in adaxial epidermal cells of V. faba cotyledons.
ingrowth wall induction (Fig. 6). A secondary burst in net 
H$_2$O$_2$ production (Fig. 2), probably accounted for by an up-
regulated ethylene-insensitive expression of VfrbohC (Fig. 3C;
Table 1), occurred concurrently with initiation of WI
formation (see Wardini et al., 2007). Thus the two consecutive
bursts in net ROS production (Fig. 2) probably perform
differing functions in ingrowth wall formation. A hint that
this is so is demonstrated by differing Ca$^{2+}$ dependencies of
the two ROS bursts (Fig. 2) and by the finding that only
VfrbohA expression is ethylene sensitive (Fig. 7) as reported
for ethylene-sensitive expression of BO-RBOHD (Jakubowicz
et al., 2010) and a maize rboh homologue (Rajhi et al., 2011).

ROS function as a polarizing signal for uniform wall
formation

DAB staining of cultured cotyledons revealed that H$_2$O$_2$
production was localized to trans-differentiating adaxial
epidermal cells, with the H$_2$O$_2$ signal polarized to their
outer periclinal walls (Fig. 4A). Moreover, this result
demonstrates that a large proportion of extracellular H$_2$O$_2$
(Fig. 2) was located in the outer periclinal walls of adaxial
epidermal cells (Fig. 4A) where the ingrowth wall is
deposited (McCurdy et al., 2008; and also see Fig. 5B). A
strict spatial linkage between extracellular ROS and ingrowth wall formation was demonstrated by concurrence of
these two phenomena under conditions of an experimentally
imposed blockage of ROS accumulation (Fig. 4B versus
Fig. 5C, respectively) or delocalization of the extracellular
ROS signature (Fig. 4C versus Fig. 5D–I). Significantly,
a delocalized ROS signature induced deposition of a uni-
form wall on all epidermal cell interfaces but WI formation
was restricted to their outer periclinal walls alone (Fig. 5D–
I). This suggests that ROS regulate polarized formation of
the uniform wall but may not play a direct role in
subsequent WI deposition. How ROS act to direct forma-
tion of the uniform wall is unknown.

Coordinate regulation of induction of trans-
differentiation by ethylene, glucose, and ROS

WI induction is regulated by auxin (Dibley et al., 2009) and
ethylene (Zhou et al., 2010), with glucose functioning as
a gate-keeper determining whether an ethylene cascade
proceeds (Andriunas et al., 2011). \( \text{H}_2\text{O}_2 \) is now added to this regulatory pathway (this study). In this context, several lines of evidence suggest that \( \text{H}_2\text{O}_2 \) exerts its regulatory effect downstream of ethylene as it does during leaf abscission signalling (Sakamoto et al., 2008) and adventitious root outgrowth (Steffens and Sauter, 2009). For instance, enhancing ethylene biosynthesis could not recover DPI-mediated suppression of WI induction (Fig. 6A). In contrast, \( \text{H}_2\text{O}_2 \) partially recovered repression of WI induction mediated by reduced ethylene biosynthesis (Fig. 6A), consistent with operation of two routes for ethylene action on WI formation, one of which is ROS dependent. The latter appears to be mediated through ethylene positively regulating \( \text{H}_2\text{O}_2 \) production (De Cnodder et al., 2005) prior to, and during, ingrowth wall induction (Fig. 6B). At least for the period prior to WI induction, ethylene-dependent regulation of \( Vf\text{rbohA} \) transcription (Fig. 7A) accounted for ethylene-enhanced \( \text{H}_2\text{O}_2 \) production (Fig. 6B). A finely tuned homeostasis of ethylene/\( \text{H}_2\text{O}_2 \) regulation (Jakubowicz et al., 2010) is indicated by a ROS feedback on ethylene biosynthesis within the trans-differentiating epidermal cells (Fig. 7D).

Integral to induction of an ingrowth wall in cotyledon epidermal cells is polarization of the \( \text{H}_2\text{O}_2 \) signature to their outer periclinal walls (Fig. 4A). Sensitivity of the \( \text{H}_2\text{O}_2 \) signature to DPI (Fig. 4B) supports the claim that flavin-containing enzymes, such as NOX, were required to synthesize the polarized \( \text{H}_2\text{O}_2 \) signature. Planar localization of transporter proteins has been reported recently in plasma membranes of epidermal and endodermal cells of roots, but the signal(s) responsible for imposing planar polarity are yet to be identified (Grebe, 2010). An interaction between auxin and ethylene is responsible for planar positioning of root hairs of epidermal cells (Fischer et al., 2007). Whether such a regulatory mechanism operates within trans-differentiating epidermal cells will be the subject of a further investigation.

Supplementary data

Supplementary data are available at JXB online.

**Figure S1.** Verification of WI induction and response to diphenyleneiodonium (DPI) following culture of *Vicia faba* cotyledons on MS medium containing the hydrogen peroxide stain, diaminobenzidine (DAB).

**Figure S2.** SEM images illustrating the effect of diphenyleneiodonium (DPI) on WI induction in *Vicia faba* cotyledons.

**Figure S3.** Effect of ascorbic acid (AA), glutathione (GSH), and catalase on temporal changes in extracellular ROS production during culture of *Vicia faba* cotyledons.

**Figure S4.** Amino acid sequence alignment of *Vicia faba* respiratory burst oxidase homologues (rbhs) with the catalytic subunit of human phagocytic respiratory burst oxidase gp91phox.

**Figure S5.** Phylogenic analyses of *VfrbohA* and *VfrbohC* in relation to other respiratory burst oxidase homologues (rbhs) from various species.

**Table S1.** Primer sequences used for cloning and real-time PCR of *Vicia faba* respiratory burst oxidase homologue (*Vfrboh*), and *Vicia faba* elongation factor \( \alpha \) (*VfEF\( \alpha \*)) genes.

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