Journal of Experimental Botany, Vol. 59, No. 13, pp. 3661–3674, 2008
doi:10.1093/jxb/ern214 Advance Access publication 5 September, 2008
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Expression profile analysis of genes involved in cell wall regeneration during protoplast culture in cotton by suppression subtractive hybridization and macroarray

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Received 11 May 2008; Revised 27 July 2008; Accepted 30 July 2008

Abstract

The molecular mechanisms underlying cell wall biosynthesis are poorly understood. In this study, microscopic analysis showed that protoplasts generated a new cell wall within 48 h after transfer to a wall-regeneration medium. To identify genes related to cell wall biosynthesis in cotton, suppression subtractive hybridization was used to visualize differential gene expression at seven time points within the first 48 h. In total, 412 differentially expressed sequence tags (ESTs; >3-fold) were identified, and 210 unigenes were sequenced successfully. As confirmed by reverse-transcription PCR (RT-PCR) and real-time quantitative reverse-transcription PCR (QRT-PCR) analysis, the selected genes displayed complex expression patterns during cell wall regeneration from protoplasts and included most previously published cell-wall-associated genes. ESTs similar to cell-wall-protein genes, such as proline-rich protein (*PRPL*), glycine-rich protein (*GRP*), extension (*EPR1*), fasciclin-like arabinogalactan protein (*FLA2*), and expensing-like protein (*EXLA* and *EXLB*), which might participate in primary cell wall or secondary cell wall construction and modification, were up-regulated during cell wall regeneration from protoplasts. Sucrose synthase, an important enzyme in the sugar signalling pathway, played important roles in cellulose biosynthesis. Our findings also highlighted the function of some transcription factors during cell wall regeneration from protoplasts, including the squamosa promoter binding protein-like 14 (SPL14), NAC, Gbiaa-re, MYB, WRKY, swellmap 1 (SMP1), RAD5, and zinc finger family protein, as well as the enrichment of Ca^{2+}-calmodulin signal molecules. On the basis of the gene expression profiles, a model of cell wall regeneration from protoplasts derived from cotton suspension cultures is proposed.

Key words: Cell wall regeneration, cotton, expression pattern, suppression subtractive hybridization.

Introduction

The plant cell wall is a dynamic structure, chiefly composed of complex polysaccharides, proteins, and aromatic substances. Individual cells are characterized by their respective cell wall structures, which serve key roles across a wide spectrum of biological processes, including cell growth and differentiation as well as response to abiotic stress and attack by other organisms (Cosgrove, 2001). These cell walls occur in certain cell types, such as fibres, vessels or tracheids. During cell growth, these cells are surrounded only by primary walls, which are mainly composed of polysaccharides and proteins. However, these cells synthesize a secondary wall when cell growth ceases (Carpita and McCann, 2000). The compositions of certain cell walls vary depending on the plant species, tissue type, cell type, region within the cell wall, and developmental

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Abbreviations: CIPK9, CBL-interacting protein kinase 9; COMT, caffeic acid *O*-methyltransferase; EPR1, extension proline-rich 1; EST, expressed sequence tag; EXL, expansin-like; FLA, fasciclin-like arabinogalactan protein; GRP, glycine-rich protein; GST, glutathione-*S*-transferase; LTP, lipid-transfer protein; Mn-SOD, manganese superoxide dismutase; MPPBETA, metalloendopeptidase; PIP, plasma intrinsic protein; PRP, proline-rich protein; QRT-PCR, real-time quantitative reverse-transcription PCR; RT-PCR, reverse-transcription PCR; SMADC, *S*-adenosylmethionine decarboxylase; SMP1, swellmap 1; SPL14, squamosa promoter binding protein-like 14; SSH, suppression subtractive hybridization; SUS2, abnormal suspensor 2; TCTP, translationally controlled tumour protein; TIP, tonoplast intrinsic proteins; XTH, xyloglucan endotransglucosylases/hydrolases.

stage of the cell. Cell wall composition can also be influenced by genetic variation within a species, growth conditions, and age of the plant (http://www.epobio.net/workshop0605/foundationpaper_plantcellwalls.pdf.).

While the complexity and diversity of plant cell walls have hampered dissection of the molecular processes by which the wall structures are generated, assembled, and remodelled during growth and differentiation of an organ, the structural complexity and diversity are reflected by a vast array of proteins involved in their construction and disassembly processes, which make up approximately 10% of the cell wall mass and are typically encoded by large gene families (Chivasa *et al*., 2002). During the last decade, significant progress has been made in the identification of important enzymes and proteins responsible for the construction and modification of the cell wall framework, including cellulose synthase and xyloglucan endotransglucosylases/hydrolases (XTH) (Fry, 1995; Wojtaszek, 2000), as well as certain cell wall proteins such as expansin (Cosgrove, 2001).

Proteomic approaches have been adopted to gain insight into the function and localization of individual cell wall proteins *in muro*, which allows the large-scale analysis of protein expression profiles, including their post-translational modifications, and protein–protein interactions (Pandey and Mann, 2000). Sotiriou and Spyropoulos (2002) incorporated radioactivity in the regenerated cell wall to study the galactomannan biosynthesis by protoplasts. Kwon *et al*. (2005) employed two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization-time of flight/mass spectrometry to map and identify 71, 175, and 212 proteins in three pools. The analysis revealed a set of enzymes specifically involved in cell wall expansion and construction in suspension-cultured cells. This approach has also revealed a set of cell wall proteins that had not been predicted to be localized in cell wall spaces. Nuclear magnetic resonance spectroscopy was developed that allows the fingerprinting and structural analysis of the whole cell wall to characterize the lignin and carbohydrate components in cell walls (Ralph and Lu, 2004).

Besides those proteomic approaches mentioned above, other methods have also been used to understand the mechanism of gene regulation during cell wall construction. Reverse genetic approaches based on oligo DNA microarray technology coupled with phenotypic analyses of T-DNA insertion lines have given some insight into understanding the functions of a large number of genes involved in the cell wall (Bonetta *et al*., 2002; Manfield *et al*., 2004). Oomen *et al*. (2003) investigated primary cell wall biosynthesis using cDNA-AFLP with potato leaf protoplasts; their findings suggested that the active regeneration of a new cell wall did not result in markedly increased expression of genes involved in cell wall biosynthesis or modification. Imoto *et al*. (2005) manufactured a gene-specific 70-mer oligo microarray consisting of 765 genes classified into 30 putative families of proteins that are implicated in the cell wall dynamics of *Arabidopsis*. Using this array system, they identified several sets of genes expressed differentially at specific growth stages of the inflorescence stem, indicating that there is a division of roles among family members within each of the putative cell-wall-related gene families. Collén *et al*. (2006) identified numerous genes potentially involved in the construction of the cell wall or the extracellular matrix in red algae. However, only a limited number of cell wall genes, particularly in terms of cell wall dynamics, compared with those predicted numbers based on the genomic database, have been characterized (Girke *et al*., 2004), and few studies have examined the regulation genes during cell wall biosynthesis.

More and more genes involved in cell wall biosynthesis have been identified from different plant species. The model plant system *Arabidopsis* has been widely used for the availability of genome sequences and the large amount of gene expression data (Yokoyama and Nishitani, 2006). In addition, mutant analyses have identified many genes related to cell wall biosynthesis (Zhong *et al*., 1997; Cano-Delgado *et al*., 2000; Fagard *et al*., 2000; Turner *et al*., 2001). Poplar emerged as an additional model species for analysing secondary wall biosynthesis since its genome has been sequenced and large EST databases are already available (Hertzberg *et al*., 2001). In addition, the *Zinnia* cell culture system was an excellent tool to study wall biosynthesis (Demura *et al*., 2002). The cotton fibre is a differentiated single epidermal cell, the development of which has four overlapping stages that are mainly for cell wall synthesis. Systematic molecular and genomic surveys of the genes crucial for the fibre development process have presented a lot of data about the genes associated with cell wall synthesis (Arpat *et al*., 2004; Shi *et al*., 2006).

Protoplasts are regarded as particularly suitable for studying cell wall biosynthesis (Burgess, 1983). Although early findings suggested that protoplasts are not able to rebuild a normal cell wall (Pilet *et al*., 1984; Gould *et al*., 1986), later work showed that they retain the capacity to develop a normal cell wall under suitable culture conditions (Shea *et al*., 1989). These previous studies investigated the biochemical and cytological characteristics of protoplasts by observing their newly generated cell walls. In this study, cotton protoplasts are used with suppression subtractive hybridization (SSH) to isolate the genes involved in cell wall biosynthesis that are switched on by regenerating protoplasts, and the expression patterns of candidate genes are analysed.

Materials and methods

Cell wall regeneration from protoplasts

Protoplasts were isolated from *Gossypium hirsutum* L. Coker 201 embryogenic calli and transferred to a wall-regeneration medium described by Yang *et al*. (2007). Aliquots were taken at various

time points (0, 3, 6, 12, 18, 24, 36, 48, and 72 h) and observed using fluorescence microscopy with staining of the cellulose by 0.001% Calcofluor White M2R (Sigma, St Louis, MO, USA) at 25 °C for 5 min. Both ordinary light images and fluorescent images for individual specimens were observed under a fluorescence microscope (DM2500, Leica, Wetzlar, Germany) equipped with a UV fluorescence filter set (excitation filter, 350 nm; barrier filter, 430 nm). The images were then photographed with a digital camera (DFC300 FX, Leica, Wetzlar, Germany).

SSH library construction

To identify the genes involved in cell wall biosynthesis, cells at various stages (0, 3, 6, 12, 18, 24, and 48 h) within the first 48 h after culture were used for RNA isolation. Total RNA was isolated from each sample using a modified guanidine thiocyanate method based on the protocols of Salzman *et al.* (1999). Following extraction, the RNA sample was dissolved in 200 µl diethyl pyrocarbonate-treated water. RNA samples were quantified by absorbance at 260 nm and evaluated on an agarose gel. The PCR-Select subtractive library was constructed using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the method of Zeng *et al.* (2006). Driver cDNA was synthesized from the RNA from fresh protoplasts, and tester cDNA was produced from mRNA isolated at the various time points after culture. After tester cDNA was digested with *Rsa*I and ligated to adaptor 1 and 2R, two rounds of hybridization and PCR amplification were performed to enrich the differentially expressed sequences. The second round PCR products of subtracted cDNA were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and then transformed into *Escherichia coli* DH5α. Positive transformants based on blue/white colour selection were cultured overnight in 384 well microplates. PCR was performed with universal primer T7/SP6 complementary to the plasmid vector sequences flanking the insert cDNAs (T7 primer, 5′-TAATACGACTCACTATAGGG-3′; SP6 primer, 5′-ATTTAGGTGACACTATAG-3′) using 1 µl bacterial overnight cultures as template. PCR products (5 µl) were evaluated on 1.2% agarose gel to analyse the length of the product of each clone.

Differential screening and expression pattern analysis

All PCR products were precipitated by the addition of 100 µl anhydrous ethanol and were resuspended in 20 µl ddH_2O. After analysis by agarose-gel electrophoresis and spectrophotometric quantification, the PCR products from each of the recovered clones of the subtractive library was transferred to 384 well microplates and dotted onto Hybond-N^+ nylon membranes (Amersham International, Little Chalfont, Bucks, UK), using a Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA). The filter (8×12 cm) was arrayed into 384 grids, each containing four dots. Cotton polyubiquitin, actin, and histone ESTs were printed as internal controls and ddH_2O as negative controls. After air drying, membranes were denatured in 0.6 M NaOH for 2 min, neutralized in 0.5 M TRIS–HCl (pH 7.5) for 2 min, and rinsed in distilled water for 30 s. The DNAs were then permanently attached to the filters in an oven at 80 °C for 2 h, and the filters were stored at –20 °C for later use. First, the unsubtracted driver cDNA and tester cDNA labelled with [^{32}P]dCTP were used as the hybridization probe for the cDNA array analysis (Superscript II, Invitrogen, Foster City, CA, USA) to identify the differentially expressed genes, and then the cDNAs at various time points (0, 3, 6, 12, 24, and 48 h) were used as probes for further investigation of the expression patterns of some putative genes. The probes were added to the hybridization solution containing the following reagents: 5× SSC, 0.1% SDS, 50 mM TRIS–HCl, 10 mM EDTA, and 1× Denharts. Hybridization was carried out overnight at 65 °C, and the membranes were washed twice with 2× SSC and 0.1% SDS for 20 min and 0.5× SSC and 0.1% SDS for 20 min at 65 °C.

The hybridized membranes were exposed to Imagingplate (Fuji Photo Film Co., Ltd., Tokyo, Japan) for 12 h. The signals of filters were scanned using FLA-5000 Plate/Fluorescent Image Analyser (FUJIFILM Life Science, West Avenue, Stamford USA) and analysed with the software Array-Gauge Version 1.0 (Fuji Photo Film Co., Ltd.). The array was quantified after background subtraction and normalization. Differential screening and expression pattern analysis data were the average of two independent experiments, respectively. The expression profile was analysed by CLUSFAVOR 6.0 (Peterson, 2002). The intensity ratios were $\log_2$-transformed, and the clustering method was complete linkage (furthest neighbour). The results from the hybridizations were recorded for each clone, and those showing the most marked differential expression were selected for sequencing.

Sequence analysis and bioinformatics

Differentially expressed colonies identified by differential screening were randomly picked from the plated subtractive cDNA library for sequence analysis (Augct Biotechnology, Beijing, China). EST assembly was performed to obtain uniESTs using BioEdit software (http://www.bbioo.com). Homology searches of all sequences were compared to the GenBank database by using the tBLASTx algorithms at the NCBI network service (http://www.ncbi.nlm.nih.gov/BLAST/). The ESTs were named after homologous sequences in GenBank, and ESTs with BLAST E values $>10^{-5}$ were designated as having 'no significant similarity'. Functional categories were assigned according to YPD/SGD.

RT-PCR and QRT-PCR analysis

To determine the expression of candidate genes, first-strand cDNA was generated from 3 µg RNA samples by using the Superscript III RT (Invitrogen) and the products were adjusted to initial RNA concentration of 2 ng μl^{-1} for RT-PCT and QRT-PCR. Gene-specific primers were designed according to the cDNA sequences using the Primer Express Software 2.0 (Applied Biosystems, Foster City, CA, USA) and synthesized commercially (Augct Biotechnology); primers are listed in Supplementary Table S1 at *JXB* online. RT-PCR was performed in 20 µl reactions using 5 µl first-strand cDNA as template. As a control, the polyubiquitin transcripts were analysed. The general program was denaturation at 94 °C for 3 min, followed by 28 cycles of 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s, and then a final extension at 72 °C for 5 min. One to seven additional cycles were added for some lower expression genes. The PCR products (8 µl) for each sample were then electrophoresed in a 2% ethidium bromide agarose gel and viewed under ultraviolet light. For QRT-PCR, 20 µl QRT-PCR reactions were run in three duplicates on an ABI Prism 7000 Sequence Detection System and software (Applied Biosystems), using 5 µl first-strand cDNAs and SYBR Green PCR Master Mix (Applied Biosystems). Thermal cycling was performed with an initial denaturation step of 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 58 °C for 15 s, and 72 °C for 45 s. Relative quantitation of gene expression was calculated and normalized to polyubiquitin.

Accession numbers

The sequences described in the present paper have been submitted to GenBank under the accession numbers FF403627 to FF403836.

Results

Cotton protoplasts regenerate a new cell wall within 48 h

Protoplasts isolated from embryogenic calli of *Gossypium hirsutum* L. Coker 201 were transferred to culture

medium. Aliquots were taken at various points and studied using light and fluorescence microscopy with and without staining by Calcofluor White, which preferentially stains cellulose. Although the development of a new cell wall by the protoplasts did not occur in a synchronous fashion, several distinct stages could be identified. Figure 1 shows representative microscopic images of protoplasts at eight different time points. Fresh protoplasts were not stained (Fig. 1A, B), indicating that the cell walls had been completely degraded. In the early stages of culture, protoplasts showed no staining with Calcofluor White until 6 h after culture (Fig. 1C–F). Calcofluor White staining was obvious after 24 h (Fig. 1K, L), when the majority of the cells seemed to have formed a cell wall. These cells continued growth (Fig. 1M, N) and after a few hours the first cell divisions appeared (Fig. 1O, P). Thus, the biosynthesis of cell wall occurred during the first 48 h, mainly within the first 24 h. Therefore, cDNAs from fresh protoplasts were used as driver and cDNAs harvested from different points (3, 6, 12, 18, 24, 36, or 48 h) as tester for SSH library construction.

Differential screening of genes identified by SSH

To identify genes differentially expressed during cell wall regeneration from cotton protoplasts, an SSH library was constructed at different stages of cell wall regeneration (see Supplementary Fig. S1 at *JXB* online). In total, 1520 clones were randomly selected following cDNA subtraction and ligation of amplified products. Macroarrays were employed to screen the subtracted libraries to identify genes preferentially expressed during cell wall regeneration (see Supplementary Fig. S2 at *JXB* online). Although PCR-selected cDNA subtraction is a powerful tool for identifying differentially expressed genes, subtractive products may contain some cDNA that are common to or have similar levels in all tissues. Detailed studies on the successful application of SSH show that, for effective enrichment of differentially expressed genes, the concentration ratio of these differentially expressed genes needs to be more than 3 (Zeng *et al*., 2006). Based on macroarray screening, cDNAs were identified that were differentially expressed in fresh versus cultured protoplasts. In total 412 clones were identified from the

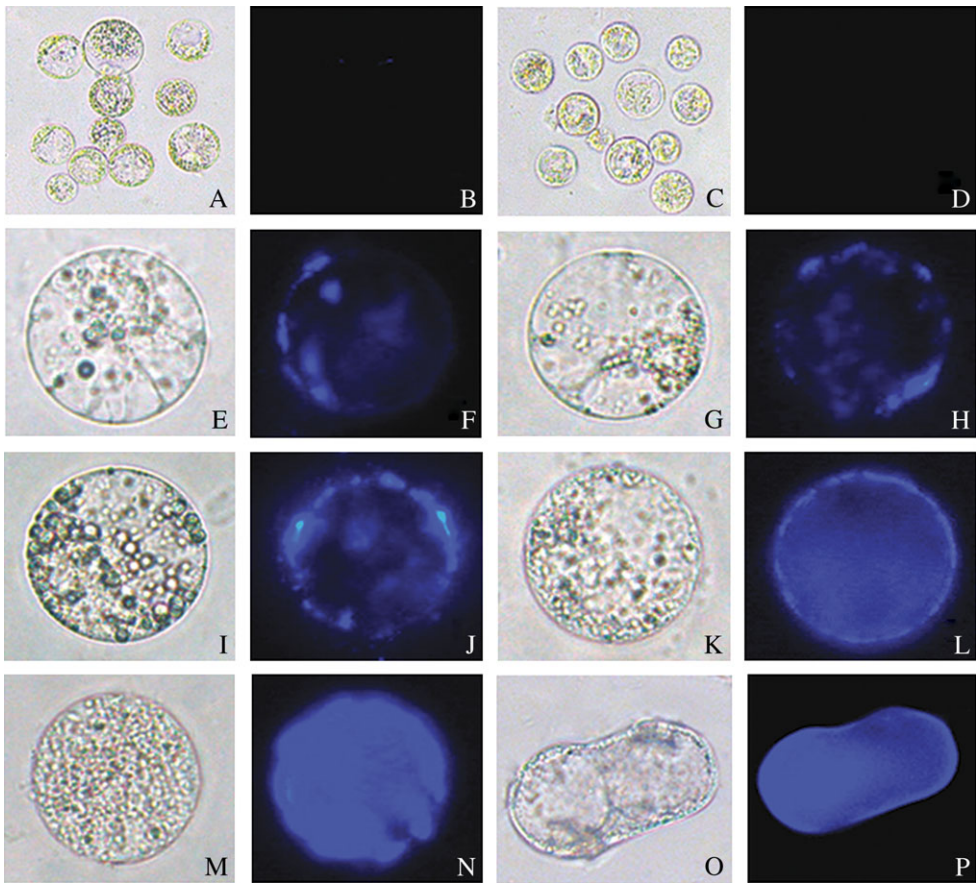

Fig. 1. Cell wall regenerated in the first 48 h of protoplast culture. Protoplasts were stained with Calcofluor White and subsequently studied with normal light (A, C, E, G, I, K, M, O) and UV light (B, D, F, H, J, L, N, P). Samples were taken at different time points after transfer to the culture medium: 0 h (A, B), 3 h (C, D), 6 h (E, F), 12 h (G, H), 18 h (I, J), 24 h (K, L), 36 h (M, N), 48 h (O, P).

SSH libraries based on initial macroarray screening; these were either expressed only in the tester or at higher levels in the tester than in the driver.

The nucleotide sequences of the 412 selected clones were analysed, and their putative functions were identified by tBLASTx searches. In total, 328 sequences were successfully sequenced, with lengths from 200 bp to 800 bp. After clustering and EST assembly, 210 (64%) unique sequences were obtained. These 210 non-redundant ESTs could be classified into three groups based on the BLAST search results. The first group consists of 148 (70.5%) ESTs with high homologies to known genes (tBLASTx expectation values [E] of $<e^{-5}$), suggesting that they are either the same gene or belong to the same gene family. The second group of 24 (11.4%) ESTs showed high similarity to unknown or hypothetical proteins. The remaining 38 (18.1%) ESTs have no homologies with any genes in the database, which might represent either previously uncharacterized sequences or fragments that are too short to reveal any significant identity. These 62 unknown ESTs may indicate that a large number of genes involved in cell wall biosynthesis were not exploited. The BLAST search results of 210 ESTs are presented in Supplementary Table S2 at *JXB* online.

Functional categories of differentially expressed genes

The significant responsive genes were classified into functional categories according to YPD/SGD (Fig. 2; see Supplementary Table S2 at *JXB* online). The main functional groups of up-regulated genes are related to metabolism and energy, including the metabolism of carbohydrates, amino acids, lipids, and nucleotides. From the group of 25 genes related to cell walls, two subgroups can be highlighted. The first subgroup comprises 16 ESTs with homologies to genes encoding cell wall proteins, including extension (*EPR1*), proline-rich proteins (*PRPL*), glycine-rich proteins (*GRP*), expansins (*EXLB1, EXLA2*), fasciclin-like arabinogalactan proteins (*FLA2*), lectin family, and nodulin (Showalter, 2001). The second subgroup includes some enzymes, such as sucrose synthase, laccase, and caffeic acid *O*-methyltransferase (COMT). These ESTs encoded some enzymes in the biosynthesis pathway of cell wall polymers. Twenty-four ESTs similar to stress-responsive genes, such as heat-shock-related protein (*DNAJ*), osmotin (*GhOSMII*), universal stress protein (*usp*), dehydrin, metallothionein-like protein (*met-l*), manganese superoxide dismutase (Mn-*SOD*), and wound-induced protein were isolated. Some ESTs related to transport were also isolated in this library. Among them were two ESTs encoding tonoplast intrinsic proteins (*TIP*) and plasma intrinsic protein (*PIP*), two water-channel genes specifically expressed during late fibre development in cotton (Liu *et al*., 2006), and two ESTs similar to ABC-transporter genes. Moreover, 13 (6.2%) of the differentially expressed ESTs were found to be involved in transcriptional regulations. Some transcription factors identified here, such as MYB, WAKY, Gbiaa-re, NAC, swellmap 1 (SMP1), squamosa promoter binding protein-like 14 (SPL14), abnormal suspensor 2 (SUS2), and zinc finger family proteins, were not previously confirmed as differentially expressed during cell wall development. Further investigation of these ESTs might reveal their function on cell wall biosynthesis. Another 13 ESTs identified were related to signal transduction, including some calmodulin (CaM)-binding protein and protein kinase. However, few of them were identified as cell-wall-associated genes. Some genes may be involved in the assembly of the extracellular matrix, as homologues to protein–protein interaction proteins containing translationally controlled tumour protein (*TCTP*), ubiquitin, metalloendopeptidase (*MPPBETA*), ubiquitin–protein ligase, and protein binding factor with domain von Willebrand factor type A.

Expression profile of ESTs involved in cell wall regeneration from protoplasts

To survey the genes preferentially expressed during cell wall regeneration from protoplasts, the array was hybridized with the cDNAs harvested at different time points

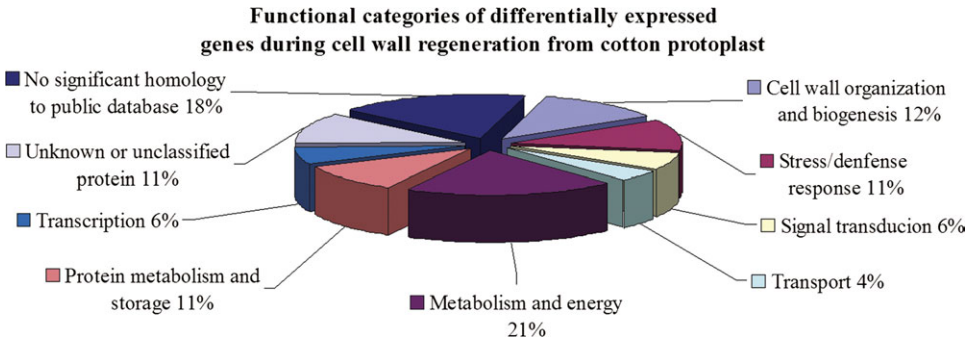

Fig. 2. Functional categories of differentially expressed genes included in cell wall biosynthesis cDNAs library according to YPD/SGD classification.

(see Supplementary Fig. S3 at *JXB* online). After being quantified and normalized, the expression profile was analysed (Fig. 3). The clones sequenced in this study showed four expression types.

Type I (138 ESTs), which includes four clusters, was expressed during the initial stage of cell wall biosynthesis and reached the highest levels around 3–6 h. The first cluster included 54 ESTs that were expressed at a high level at 3 h and then gradually decreased. These included ESTs encoding CCCH-type zinc finger protein, TIP, EXLA2, and BIK1. The second cluster included 31 ESTs that reached a high level at 3 h, decreased at 12 h, and then increased. These ESTs were homologous to SPL14, CP5, nodulin, universal stress protein, C3HC4-type zinc finger protein, Gbiaa-Re, and MYB. The third cluster included 14 ESTs that reached a peak at 6 h and then decreased. This cluster included ESTs similar to EXLB1, ENOD8, AtMRP3, and EPR1, as well as some unknown ESTs. The fourth cluster included 39 ESTs which reached another peak value at another time point. Among them were two ESTs encoding curculin-like lectin family proteins expressed at a high level at 48 h and two ESTs similar to alcohol dehydrogenase genes that reached the highest expression level at 24 h.

Type II (29 ESTs) was expressed during the middle stage of cell wall biosynthesis. These ESTs included PRP, GRP, ANAC016, ATB2, PUX4, SKP1, calcium-binding EF hand family protein, peroxiredoxin, PDR-like ABC-transporter, and cystatin.

Type III (13 ESTs) was mainly expressed at the late stage and reached the highest level around 48 h. ESTs encoding CBL-interacting protein kinase 9 (CIPK9), FLA2, SUS2, laccase, fibre quinone-oxidoreductase, and *o*-methyltransferase belonged to this type. These genes might be involved in the construction of the secondary cell wall and the initiation of cell division.

Type IV (30 ESTs) remained at a high level throughout the cell wall biosynthesis. Representative ESTs were similar to *S*-adenosylmethionine decarboxylase (SMADC), osmotin, and actin.

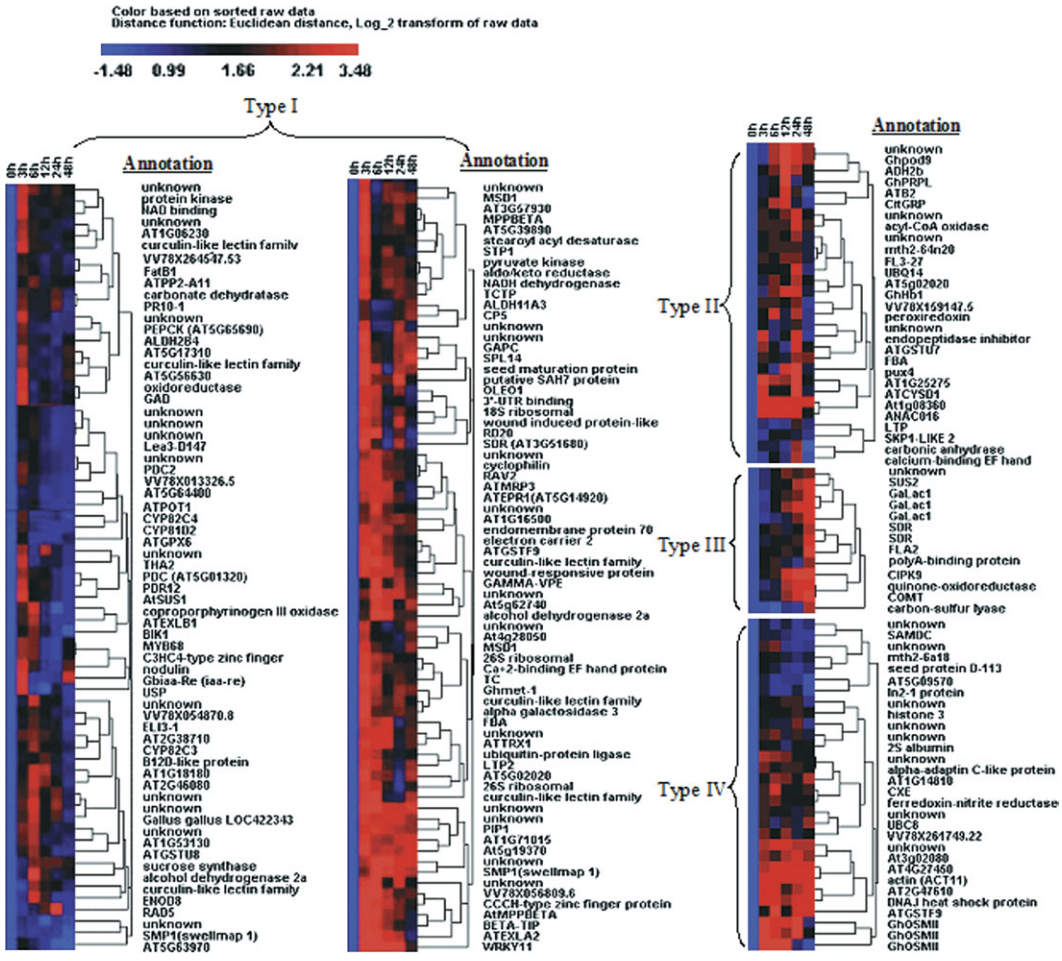

Fig. 3. Clustering of cell-wall-associated genes during protoplast development. The intensity ratios were $\log_2$-transformed, and the clustering method was complete linkage (furthest neighbour). The results from the hybridizations were recorded for each clone, and those showing the most marked differential expression were selected for sequencing. The amount of the expression ratio is indicated by the scale bar.

Confirmation of SSH expression patterns by RT-PCR and QRT-PCR analysis

To test the reliability of SSH further and to compare the expression patterns of differentially expressed ESTs among fresh protoplasts and protoplasts at different time points, RT-PCR analysis was conducted for a subset of 23 ESTs representing different expression patterns (Type I–IV) which had been identified by differential screening based on macroarray (Fig. 4). Among the 23 ESTs there were three possible cell wall structural family proteins with different expression patterns during the cell wall regeneration: PRP only expressed at a high level during the middle stage of cell wall regeneration, GRP expressed throughout the 48 h but with the highest expression level at about 12 h, and FLA2 was gradually up-regulated and expressed the highest level at the late stage. The ESTs encoding protein kinase, TIP, and nodulin were only expressed at 3 h or 6 h, indicating that these genes might relate to the initiation of cell wall biosynthesis. The EST encoding an IAA-response protein, which showed a similar expression pattern as transcription factor NAC, was up-regulated during the early and late stages of cell wall biosynthesis. An EST similar to *pux4* expressed in the embryogenic calli and during the late stage of cell wall regeneration might be related to the cell cycle. The differential expression was observed mostly at a quantitative level. Except three ESTs (NAC, ATB2 and OSM), all the other ESTs selected gave the same expression pattern as revealed by macroarray, confirming the expression pattern of 87% (20 out of 23) SSH-derived ESTs showed significant expression differences between fresh protoplasts and cultured protoplasts.

QRT-PCR was further applied to validate the expression pattern of 14 selected ESTs with putative functions involved in a diverse set of biological pathways (Fig. 5). Nine out of the 14 belonging to Type I (grouped by macroarray) showed two different expression patterns as revealed by Fig. 5A (five ESTs) and Fig. 5B (four ESTs). Among them, two ESTs encoded possible zinc family proteins. The C3HC4-type zinc finger family protein

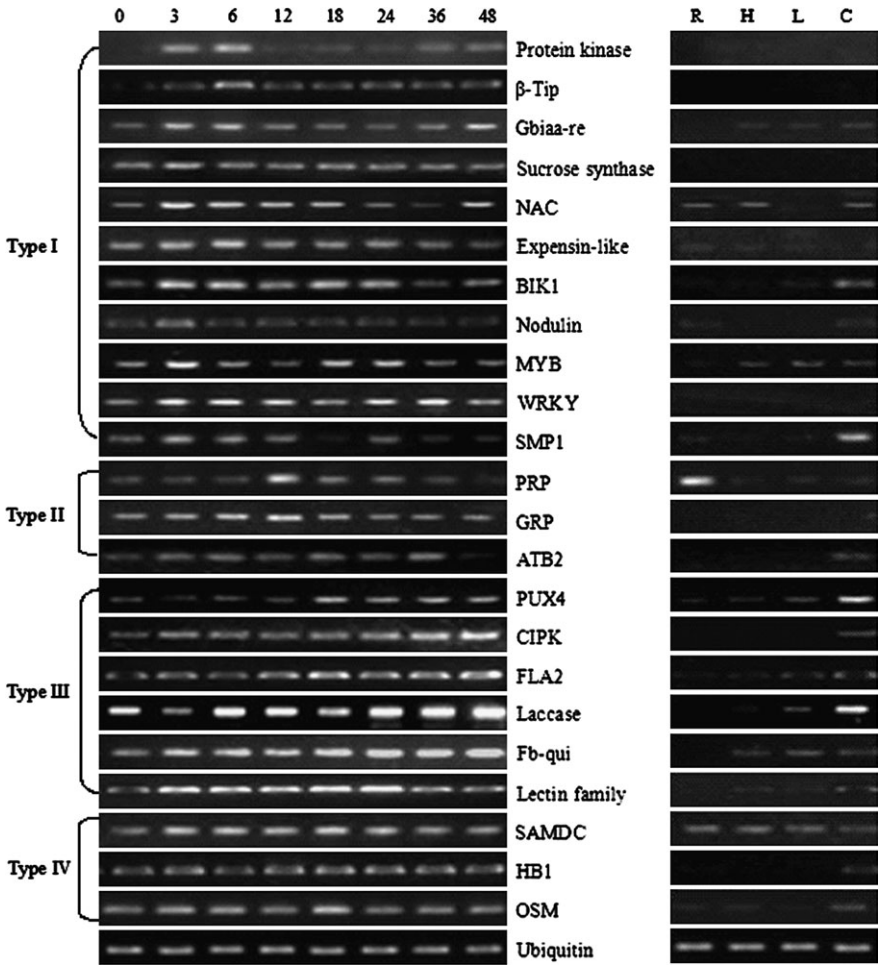

Fig. 4. Conformation of the expression pattern of some selected ESTs by RT-PCR. R, cDNAs from the root of Coker 201; H, cDNAs from the hypocotyls of Coker 201; L, cDNAs from the leaf of Coker 201; C, cDNAs from the embryogenic calli of Coker 201. The values 0, 3, 6, 12, 18, 24, 36, 48 represent the number of hours of the protoplast culture from which cDNAs were harvested.

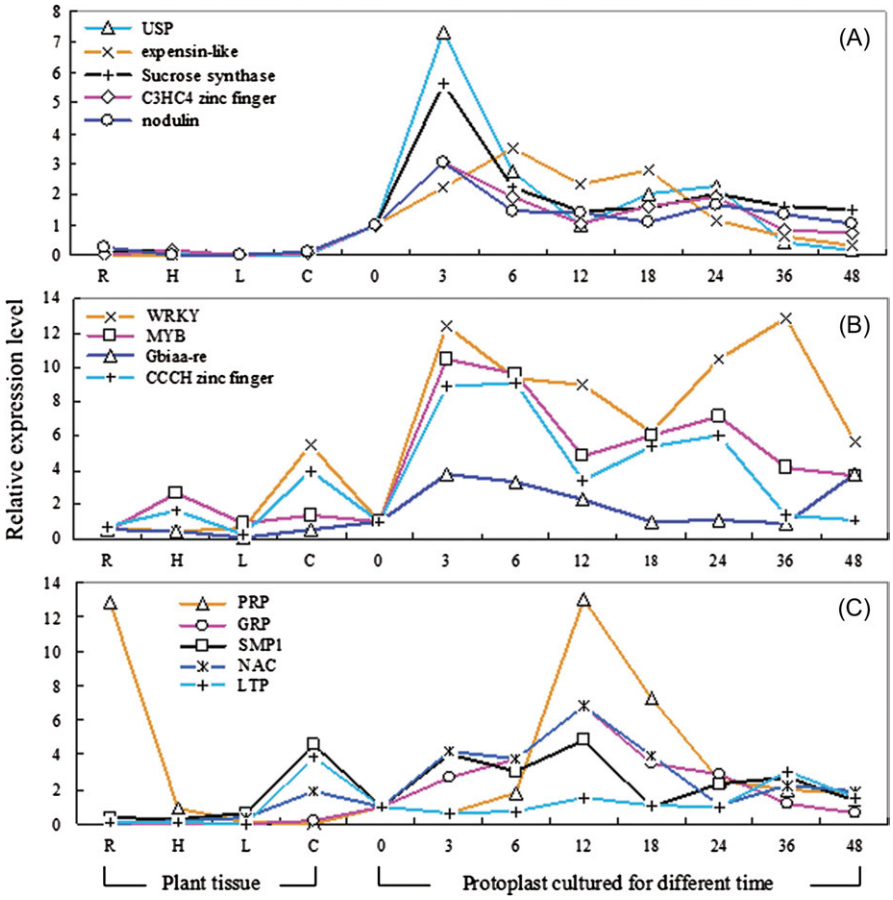

Fig. 5. The expression patterns analysis of some selected ESTs by QRT-PCR. Nine ESTs in Type I (A, B) and four ESTs in Type II, and one EST in Type III (C). R, cDNAs from the root of Coker 201; H, cDNAs from the hypocotyls of Coker 201; L, cDNAs from the leaf of Coker 201; C, cDNAs from the embryogenic calli of Coker 201. The values 0, 3, 6, 12, 18, 24, 36, 48 represent the number of hours of the protoplast culture from which cDNAs were harvested.

expressed only during the initiation of cell wall biosynthesis (Fig. 5A). By contrast, the CCCH-type zinc finger protein showed a high level for several hours during cell wall biosynthesis initiation and maintained a relatively high level over the whole of the culture period (Fig. 5B). The EST encoding a sucrose synthase gave relative quantitative differences at different time points, better in detecting expression than RT-PCR (Fig. 5A). Figure 5C contained two expression types (grouped by macroarray) with four ESTs (PRP, GRP, SMP1, and NAC) in Type II and one EST (LTP) in Type III. QRT-PCR analyses with the exact expression data gave the similar expression patterns as revealed by RT-PCR experiments for the same ESTs. Opposite to the results of SSH, The expression levels by QRT-PCR of three ESTs encoding SMP1, C3HC4 zinc finger family protein, and GRP of protoplasts cultured for 48 h differed from the trends of SSH analysis, but most of the expression patterns of the QRT-PCR were in agreement with the macroarray ratios, with a relative coefficient R=0.854 (Fig. 6).

In order to test if the differential expressions are tissue dependent, expression profiles of the 27 cDNAs in total were analysed in roots, hypocotyls, leaves, and embryogenic calli by RT-PCR or QRT-PCR (Figs 4, 5). It was found that five (PRP, NAC, SAMDC, CCCH-type zinc finger protein, and MYB) out of the 27 ESTs tended to be differentially expressed in at least one tissue, and all the other ESTs had almost no expression in root, hypocotyls, and leaf. Although 11 out of 27 ESTs which encoded possible BIK1, SMP1, laccase, NAC, MYB, ATB2, CIPK, FLA2, LTP, SAMDC, and HB1 expressed in the embryogenic calli, most of those SSH-derived ESTs were preferentially expressed during cell wall regeneration during protoplast culture.

Discussion

ESTs encoding possible cell wall proteins

Plant cell walls, the major structural component of the plant cell, also contain structural proteins. These major structural proteins include extensins, proline-rich proteins (PRPs), glycine-rich proteins (GRPs), and arabinogalactan proteins (AGPs; Showalter, 2001). In our library, some

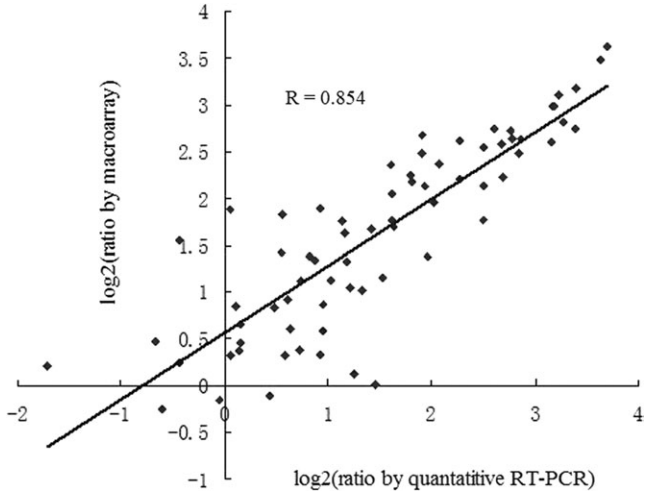

Fig. 6. Comparison of the results of QRT-PCR and macroarray for some selected genes.

ESTs homologous to genes encoding those proteins (*EPR1, PRPL, GRP*, and *FLA2*) were isolated. Extensions, synthesized in only one or a few cell types in a plant (Keller and Lamb, 1989), suggests that the proteins are functionally important parts of a particular cell wall. In our library, an EST homologous to *ATERP1* was expressed during the early stage of protoplast regeneration, indicating that this EST might be related to primary cell wall construction. Similar to the extensins, PRPs show a cell-type-specific expression pattern (Showalter, 2001). To our surprise, the ESTs isolated in this library encoding PRP protein (*PRPL*) expressed a very high level in the middle stage of protoplast regeneration as well as in roots (Fig. 5C). Unfortunately, the short length (185 bp) made it difficult to analyse the exact structure of this gene. The full-length gene will be required for further study. GRPs have been found in the cell walls of many higher plants and form a third group of structural protein components of the wall. Similar proteins likely to be cell wall proteins were found in *Arabidopsis*, rice, barley, tomato, and other plant species (Condit and Keller, 1990; Ringli *et al*., 2001). GRPs are localized mainly in the vascular tissue of the plant and expressed in xylogenesis cells (Keller *et al*., 1989; Ye *et al*., 1991). The expression pattern of the EST encoding a possible GRP gene might indicate that secondary cell wall biosynthesis is initiated at that time point (Fig. 5C). Fasciclin-like arabinogalactan proteins (FLAs) are a subclass of AGPs that have, in addition to predicted AGP-like glycosylated regions, putative cell adhesion domains known as fasciclin domains (Schultz *et al*., 2000). *FLA2* is involved in development, cell adhesion, and ABA-response in *Arabidopsis* (Johnson *et al*., 2003). The RT-PCR result for the EST encoding the FLA2 protein was up-regulated during cell wall regeneration and reached the highest expression level at 48 h (Fig. 4), indicting its significant role in cell wall regeneration.

Expansins are a group of extracellular proteins that directly modify the mechanical properties of plant cell walls, leading to turgor-driven cell extension and inducing cell wall extension and expansion (Cosgrove, 2000; Darley *et al*., 2001). Expansins were initially identified by their unique ability to induce the pH-dependent extension of plant cell walls *in vitro*, by increasing polymer mobility in the cell wall and allowing the structure to slide apart during extension (McQueen-Mason and Cosgrove, 1994). To date, expansin remains the only protein demonstrated to extend cell walls *in vitro* and *in vivo*. Although the mechanism of expansin action remains elusive, studies have suggested a reversible disruption of hydrogen bonding between cellulose microfibrils and xyloglucan, resulting in a loosening of the cell wall that allows turgor-driven deformation of the cellulose/xyloglucan framework (McQueen-Mason and Cosgrove, 1994; Cosgrove, 2001). These proteins are encoded by a large multigene family (McQueen-Mason and Cosgrove, 1994), which can be divided into four subfamilies: two expansin subfamilies, EXPA and EXPB, and two expansin-like subfamilies, EXLA and EXLB (Kende *et al*., 2004). The possible *EXLA2* and *EXLB1* genes identified in this study are members of the EXLA and EXLB subfamily. Unlike EXPA and EXPB (Cosgrove, 1997), fewer biological or biochemical functions have been established for members of the EXLA or EXLB families (Kende *et al*., 2004), despite the fact that EXLA and EXLB members possess both expansin domain I and II. Thus, the present findings might imply the possible roles for *EXLA2* and *EXLB1* genes in the cell wall construction and modification, particularly in cell wall regeneration from protoplasts.

Enrichment of regulation genes and the Ca^{2+}-CaM signal pathway in cell wall regeneration from protoplast

Our library represented enrichment of 6.2% ESTs participating in transcriptional or post-transcriptional regulation. These ESTs all have very high homology with known transcription factors, including SPL14, putative CCCH-type zinc finger protein, C3HC4-type zinc finger family protein, NAC, Gbiaa-re, MYB, WRKY, ATB2, RAV2, SMP1, and RAD5. The SPL gene family represents a group of structurally diverse genes encoding putative transcription factors found apparently only in plants, which interact with DNA in a sequence-specific manner and/or in post-transcriptional/translational modifications (Cardon *et al*., 1999). The isolation of an EST with high homology to SPL14 in our library indicted that this member might regulate the development of the cell wall. WRKY is another transcription factor only found in plants that is not only related to defence, but also regulates plant development and sugar signalling (Johnson *et al*., 2002;

Sun *et al*., 2003; Lagace and Matton, 2004; Xu *et al*., 2004). As revealed in this study, the ESTs encoding WRKY expressed a low level in embryogenic calli, and reached two peaks during protoplast regeneration, one around 3 h and another around 36 h, indicating that this gene might be involved in cell wall biosynthesis initiation and cell cycle initiation (Fig. 5B). The expression pattern revealed by RT-PCR and QRT-PCR indicated that the regenerating protoplast switched on some regulation genes involved in cell wall biosynthesis (Figs 4, 5). Further experiments are required to verify the physiological function and interaction between these factors and with other genes during cell wall biosynthesis.

During the process of cell wall regeneration, various signal transduction pathways may be involved. In addition to kinases, such as protein kinase, SKP1, BIK1, and PUX4, several ESTs involved in the Ca^{2+}-CaM signal transduction system were isolated. The cell wall is the largest calcium container in plant cells. Most previous studies focused on the function of the cell wall in the Ca^{2+}-CaM signal transduction system, and early studies mostly emphasized Ca^{2+} as the structural components of the cell wall. The action of Ca^{2+} as a signal molecule participating in cell wall biosynthesis was noted by Biro *et al*. (1984) while isolating CaM from oat coleoptiles. Sun *et al*. (1994, 1995) discovered that CaM can accelerate cell wall regeneration and cell proliferation of *Angelica* protoplasts. Ca^{2+} and CaM participate in secondary cell wall biosynthesis (Demarty *et al*., 1984; Roberts and Baba, 1987; Roberts and Haigler, 1990; Kobayashi and Fukuda, 1994). The ESTs identified in this study, which encode calcium-binding EF hand family protein, CIPK, and RD20, further indicated that the Ca^{2+}-CaM signal transduction system might play a key role in cell wall regeneration. More evidence is required, however, to prove a relationship between the Ca^{2+}-CaM signal transduction system and cell wall biosynthesis.

The unexpected genes related to glycolytic metabolism, defence and transport

In cell wall-regenerating protoplasts, several glycolytic enzymes were identified that are known to catalyse the initial steps of the glycolytic pathway. The isolation of these ESTs was unexpected. However, the biosynthesis of the cell wall would require a considerable amount of carbohydrate mobilization, and several differentially expressed ESTs were found to be involved in cell wall polymer pathways. Although some enzymes have never been considered to be secreted outside of the protoplasm, several glycolytic enzymes have been classified as cell wall components in some organisms (Pardo *et al*., 2000; Chivasa *et al*., 2002; Kwon *et al*., 2005). For example, glyceraldehyde-3-phosphate dehydrogenase (At1g13440 and At3g04120) was immunologically localized in yeast cell walls (Gozalbo *et al*., 1998). Another important glycolytic enzyme, sucrose synthase, a candidate member of the cellulose synthase complex, catalyses a reversible reaction but preferentially converts sucrose into fructose and UDP-glucose in plants (Chourey and Miller, 1995). The cell wall uses sucrose for its rapid expansion and for cellulose synthesis (Haigler *et al*., 2001). Previous studies suggest that the *Sus* gene may play important roles in cell wall cellulose synthesis during cotton fibre development (Amor *et al*., 1995; Ruan and Chourey, 1998; Hong *et al*., 2001). Two ESTs that encode sucrose synthase were isolated in the present study. The results of RT-PCR and QRT-PCR indicated that sucrose synthase is mainly expressed during the early stages of cell wall regeneration in protoplasts (Fig. 5A). Although the location of these enzymes has not been fully elucidated, substantial data support the idea that some glycolytic enzymes may be secreted out of the protoplasts during the cell wall regeneration process in *Arabidopsis* (Haigler *et al*., 2001). These results suggest that these glycolytic enzymes may fulfil some unknown functions required for the cell wall regeneration of protoplasts.

Numerous stress-related sequences were found in the library, including heat-shock proteins, peroxidases, SOD, GST, cytochrome P-450, and reductases. Some defence-related proteins are directly involved in the structural organization of cell walls and take part in an active defence system. These defence proteins may be induced by various stresses that would be generated during protoplast preparation. Alternatively, proteins required for cell wall dynamics during cell wall regeneration in the protoplasts may be mediated by the same molecular components recruited as defence proteins upon a defence response. Heat-shock proteins are an important group of stress proteins; they aid in the folding of damaged and newly formed proteins as well as in preventing aggregations of proteins (Wang *et al*., 2004). Peroxidases are implicated in defence through mechanisms that include cell wall cross-linking and the generation of toxic and potentially bacteriostatic halogenated compounds, such as bromoform, methyl iodide, and trichloroethylene (Colin *et al*., 2003). Interestingly, GSTs, another important group of ESTs found in the library, may also be involved in the detoxification process of halogenated compounds (Marrs, 1996). Finally, three ESTs similar to cytochrome P-450 were identified. However, some defence-related genes were not involved in cell wall biosynthesis but were induced by the stress in the culture. The nutrition around the protoplast and the developmental stage of protoplasts might induce the expression of particular genes. Thus, protoplast systems could be used to isolate defence-related genes, and a better understanding of these genes might then help to modify the culture medium for protoplast culture.

Regeneration of cell walls from protoplasts leads to significant changes in some transporters, as reflected by

changes in the expression of nine genes coding for transporters/channels. Two ESTs with homologies to ATP-binding cassette (ABC) transporters were also identified, *pdr12* and *ATMRP3*. ABC transporters are involved in the membrane transport of a wide range of structurally and functionally unrelated compounds, such as glutathione conjugates, lipids, inorganic acids, peptides, secondary metabolites, toxins, and drugs, and they form a large protein family (Martinoia *et al*., 2002). But there is little experimental evidence concerning the physiological role of ABC transporters (Stukkens *et al*., 2005; Kobae *et al*., 2006). Another two ESTs encoding putative TIP and PIP were also isolated from the protoplast SSH library. Plasma intrinsic protein (PIPs) and tonoplast intrinsic proteins (TIPs) are subfamilies of aquaporins. Recent studies suggested that aquaporins work as water channels by facilitating the osmosis-driven permeation of water across membranes. The *Arabidopsis* genome contains 38 sequences with homology to aquaporin in four subfamilies, and the PIP subfamily includes 13 homologues (Quigley *et al*., 2001). In cotton, the first aquaporin-encoding gene was isolated by Ferguson *et al*. (1997). Smart *et al*. (1998) amplified a MIP EST fragment, and the level of MIP protein accumulation was constant throughout fibre development. Liu *et al*. (2006) identified two aquaporin genes, *TIP* and *PIP*, in the cotton fibre SSH library. In the present study, RT-PCR indicated that the two ESTs encoding putative TIP and PIP were up-regulated during cell wall regeneration from protoplasts. Their exact roles in cell wall biosynthesis require further investigation.

The use of protoplasts in the analysis of cell wall biosynthesis

When cultured on suitable medium, plant protoplasts regenerate new wall and then initiate division to form a cell cluster. It has been suggested that the regeneration of the normal cell wall is a prerequisite for the initiation of division in protoplast-derived cells. To study the process of cell wall formation on an RNA level, SSH has been used to explore the expression profiles during the process of cell wall regeneration from cotton protoplasts. Visualization of cellulose in the walls of cultured cells indicated the formation of a new wall by the viable protoplasts within 48 h after transfer to the culture medium. Previous work by Burgess (1983) and Shea *et al*. (1989) showed that protoplasts are able to generate a completely new cell wall, making this system very suitable to study cell wall biosynthesis. Because the formation of a cell wall is necessary for further development of the protoplast and subsequent cell division, it is reasonable to deduce that regenerating protoplasts switch on many genes involved in cell wall biosynthesis.

Ideally, a study of the process of cell wall biosynthesis should be carried out in a synchronous system with a clearly defined time point at which cell wall biosynthesis is initiated. Milioni *et al*. (2001) showed that the protoplast system was a highly synchronized system that allowed the selection of specific time points for transcript profiling. The choice of this system in the present study was based on the expectation that strongly enhanced cell wall regeneration would derive from markedly increased

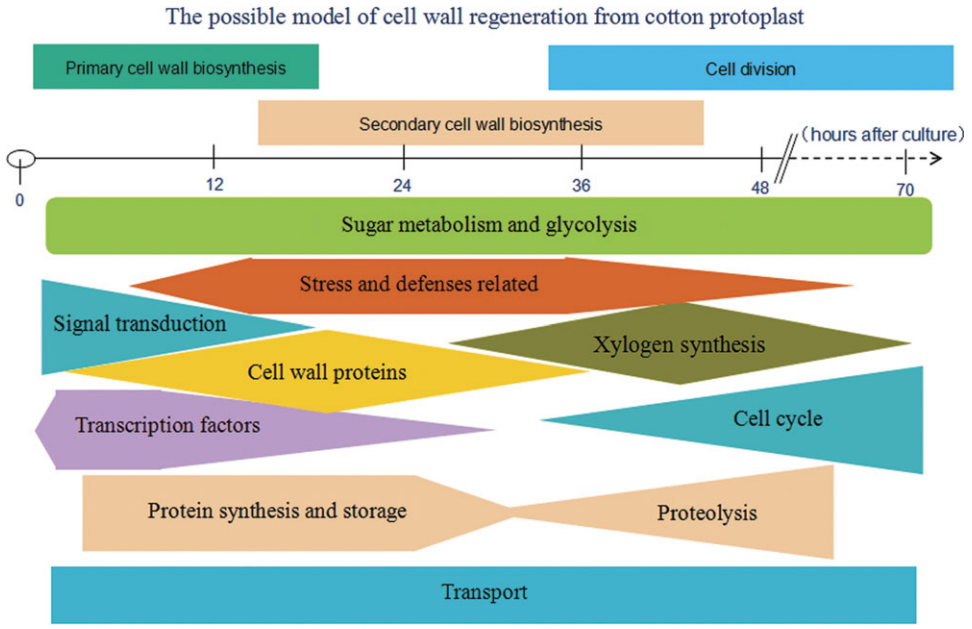

Fig. 7. Model of cell wall regeneration from cotton protoplast based on gene expression patterns.

expression levels of the genes involved, and more than 90% of protoplasts regenerated a new cell wall within 48 h of culture. Unlike Oomen *et al*. (2003), it was possible to isolate many genes related to cell wall biosynthesis. Although cellulose synthase and callose/glucan synthase were not isolated in our library, possibly because primary cell wall synthesis basically takes place in all young growth tissues, a model of cell wall regeneration in protoplasts derived from cotton suspension cultures according to the expression profile has been proposed (Fig. 7).

Supplementary data

The following materials are available at *JXB* online.

Supplementary Table S1. The primers used in RT-PCR and QRT-PCR analysis.

Supplementary Table S2. Homology analysis of differentially expressed sequences.

Supplementary Fig. S1. PCR-select cDNA subtraction. Electrophoresis results of total RNA (A), the first round of PCR product (B), the second round of PCR product (C), and the length of the inserts in the SSH library (D).

Supplementary Fig. S2. Differential screen of SSH cDNA library by tester and driver. cDNA probe from driver (A) and tester (B).

Supplementary Fig. S3. Expression pattern analysis of cDNA during cell wall biosynthesis using reverse northern blot assay. Quadruplicate colony dot blots were prepared, and the membranes were hybridized with labelled probes. cDNA probe from 0 h protoplast (A), 3 h protoplast (B), 6 h protoplast (C), 12 h protoplast (D), 24 h protoplast (E), and 48 h protoplast (F).

Acknowledgements

Support from the National Basic Research Plan (2004CB117301) and the National Cotton Production Tech-support Program (Ministry of Agriculture, China) are appreciated.

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