INTRODUCTION

Coleoptile is the pointed sheath protecting the first leaf of grass species such as maize. Since Charles Darwin first observed growth movements of the shoot tip (coleoptile) toward light (Darwin, 1880), the coleoptile has long been used as a model system to study plant growth and development (Haga & Iino, 1998).

The coleoptile growth is vital for successful seed germination and early seedling establishment. During germination, the coleoptile, as well as mesocotyl, elongates through cell expansion and pushes the shoot out of the soil or water surface. The rapid elongation of the coleoptile is induced by auxin coming from the tip, with cell number unchanged (Philippar et al. 1999). Once the coleoptile perceives light, its growth ceases and the leaves appear (Briggs, 2014; Li et al. 2013).

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The coleoptile length substantially contributes to deep-sowing tolerance in cereal plants under drought or submerged conditions (Liu et al. 2017; Magneschi & Perata, 2009; Mohan et al. 2013). Therefore, the coleoptile growth is often targeted for selecting deep-sowing tolerant cultivars.

Several hypotheses have been proposed to explain the dynamics of auxin-induced growth in plants, notably the acid-growth theory (Arusuffi & Braybrook, 2018; Rayle & Cleland, 1992), based on the evidence mostly coming from in vitro experiments using coleoptile/hypocotyl segments. The mechanisms required for plant growth are not fully clarified yet (Narsai et al. 2015), especially with respect to the proteome changes in vivo in the rapidly growing coleoptiles in model plant maize. The existence of growth-limiting proteins was proposed to explain the regulation of maize coleoptile growth (Kutschera & Wang, 2016), but the candidate proteins are speculative. In Arabidopsis, gene expression or protein synthesis was not suggested to be necessary for the initiation of cell elongation of hypocotyl segments (Schenck et al. 2010). In dark- or light-grown rye seedlings, no significant changes in protein profiles were detected during coleoptile growth (Fröhlich & Kutschera, 1995). By contrast, using in vitro cultures of rye coleoptile segments, proteomic analysis showed that the initiation of coleoptile elongation was associated with at least two auxin-responsive proteins (Deng et al. 2012), while its cessation was related to the degradation of vacuolar H^+—ATPase (Kutschera et al. 2010). Using maize coleoptile segments incubated in vitro, 15 proteins with known or predicted roles in cell wall biosynthesis were identified by proteomic analysis (Li et al. 2013). However, previous proteomic studies were almost performed with in vitro cultures of coleoptile segments, little is known about the significance of proteome changes in vivo in the rapidly growing coleoptiles. Although proteomic studies have showed protein changes during coleoptile elongation in rice under anoxic conditions (Huang et al. 2005; Sadiq et al. 2011), these results are not suitable for interpretation of coleoptile growth in other cereal plants such as maize, wheat, and barley, because their seeds lack the ability to germinate under anoxic conditions (Takahashi et al. 2011). The aim of the present work was to explore the significance of proteome changes in vivo during coleoptile growth. Using two-dimensional gel electrophoresis (2DE) combined with mass spectrometry, we investigated protein changes and identified differential abundance proteins (DAPs) that are associated with rapid growth of maize coleoptiles.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Seeds of the maize cv. Zhengdan958 were surface sterilized in 1% sodium hypochlorite for 5 min and washed thoroughly with distilled water. After 12-hr soaking, uniformly sized seeds (5 replicates, 30 each) were placed in Petri dishes having three layers of moist Whatman paper and incubated at 28°C in darkness. Coleoptiles were daily collected over 7 days after germination (DAG). The coleoptiles were excised from the basal 1 mm above the coleoptile node, and the enclosed primary leaves were removed using a pair of forceps. The coleoptile segments were used for length, fresh weight (FW), and dry weight (DW) assays (Edwards et al. 2012), light microscopy and protein analysis. Analysis of variance was performed using Student’s t test.

2.2 | Light microscopy

For preparation of paraffin sections, coleoptile segments (1–2 mm long) without the tip region were cut from the same basal region above the coleoptile node at 1.5, 3, and 5 DAG, respectively. The segments were fixed in the solution consisting of 5% formaldehyde, 5% acetic acid, and 45% ethanol for 12 hr and dehydrated in gradient ethanol solutions. The dehydrated tissues were embedded in paraffin and 10-μm thick slices were prepared with a microtome (RM-2016, Leica, German). The slices were stained with safranin/fast green (Ruzin, 1999) and recorded under a light microscope equipped with ToupView x 86 software (ToupTek Photonics, China).

2.3 | Phytohormone assay

The levels of indole-3-acetic acid (IAA), gibberellins (GAs), salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) in maize coleoptiles were assayed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) technique as described previously (Niu et al. 2019). Approximately 0.5 g (FW) coleoptile samples were powdered and extracted for 30 min at 4°C with 10 ml isopropanol/hydrochloric acid buffer. And then 20 ml dichloromethane was added and shaken at 4°C for 30 min. After centrifugation at 13,000 g for 5 min at 4°C, the organic phase was extracted and dried in N_2. The residues were resuspended in 200 μl methanol plus 0.1% methanol acid and filtered with a 0.22 μm filter membrane. The filtrate was used for HPLC-MS/MS with a ZORBAXSB-C18 (Agilent Technologies) column (2.1 × 150; 3.5 μm) at 30°C. The sample injection volume was 2 μl. The mobile phase: A: B= (methanol/0.1% formic acid): (water/0.1% formic acid); elution gradient: 0–2 min, A = 20%; 2–14 min, A increased to 80%; 14–15 min, A = 80%; 15.1 min, A decreased to 20%; 15.1–20 min, A = 20%. The MS conditions were as follows: the pressure of the air curtain, nebulizer, and aux gas was 15, 65, and 70 psi, respectively; the spray voltage was 4,500 V; and the atomizing temperature was 400°C.

2.4 | Protein extraction and determination

Coleoptile proteins were extracted with trichloroacetic acid/acetone precipitation according to the previous research (Niu, Zhang, et al., 2018). In brief, 10–20 coleoptiles were ground in an SDS-containing buffer (1% SDS, 0.1 M Tris–HCl, pH 6.8, 2 mM EDTA-Na_2, 20 mM DTT and 2 mM PMSF) and centrifuged at 15,000 g for 5 min
(4°C). The supernatant was collected and precipitated with an equal volume of cold 20% trichloroacetic acid/acetone on ice for 5 min and centrifuged as mentioned above. The protein pellets were washed twice with cold acetone and air-dried. Then, the dried pellets were redissolved in Laemmli SDS buffer for SDS–PAGE (Laemmli, 1970) or in 2DE rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer, pH 4–7, GE Healthcare) for isoelectric focusing (IEF). Bradford assay was performed to determine the protein concentration (Bradford, 1976).

2.5 | Peroxidase (POD) isozyme activity assay

Crude enzyme was extracted according to the method of Lee and Lin (1995) with some modifications. The coleoptiles (about 1 g FW) were pulverized with liquid N2 in a mortar and pestle and then homogenized with 1 ml of 10 mM phosphate buffer (pH 6.4). After centrifugation at 16,000 g for 10 min at 4°C, the supernatant was collected for soluble POD activity assay. The precipitate was washed at least five times using an equal volume of extraction buffer until no POD activity was detected in the supernatant. Then the precipitate was extracted in 1 M NaCl for 4 hr at 4°C with continuous shaking. After centrifugation, the supernatant was used for cell wall-bound POD activity assay.

The POD activity was measured using the method of López-Castillo et al. (2020) with some modifications. The POD activity was determined by the formation of tetra guaiacol from guaiacol in the presence of H2O2. The enzyme reaction solution contained 20 μl of the enzyme and 980 μl of substrate solution that was prepared by dissolving 8 mM guaiacol and 2.75 mM H2O2 into the 10 mM phosphate buffer (pH 6.4). The absorbance at 470 nm was read using an ultraviolet spectrophotometer (Shimadzu UV-2550, Kyoto, Japan). One enzyme unit (U) was defined as a change in absorbance of 0.01 in 1 min (Tao et al. 2018).

The soluble POD isozymes were resolved with 7.5% native gels and visualized in situ by staining with 3,3’-diaminobenzidine for 10 min at room temperature (Daud & O’Brien, 2012).

2.6 | SDS–PAGE and immunoblot analysis

Proteins were resolved by SDS–PAGE using 12.5% polyacrylamide gels. Gels were stained with 0.1% Coomassie brilliant blue (CBB) R350 or blotted onto polyvinylidene fluoride membrane with a semi-dry transfer system (Trans-Blot, Bio-Rad, USA) following the manufacturer’s instructions. After soaking in TBST buffer (50 mM Tris–HCl, pH7.5, 0.15 M NaCl, 0.1% Tween-20) containing 5% skimmed milk for 1 hr, protein blots were incubated with specific polyclonal antibodies (1:5,000 dilution) against actin, tubulin, expanse (Abbkine, USA) for 1 hr, respectively, and then incubated with POD-conjugated goat anti-mouse IgG (1:5,000 dilution, CWBIO, China) for 1 hr (Niu et al. 2019). After washing with TBST, the blots were detected with chemiluminescent HRP substrate (Immobilon™ Western, Millipore Corporation, Billerica, USA), and the images were captured with a chemiluminescence/fluorescence image analysis system (Tanon 5200, Shanghai, China). The band intensity was quantified with ImageJ program (version 1.52, Rueden et al. 2017).

2.7 | 2DE and protein identification

Proteins samples (600 μg in 200 μl) were loaded into IPG strips (11 cm, linear pH 4–7, Bio-Rad) by passive rehydration overnight at room temperature. IEF and subsequent SDS–PAGE were performed as previously described (Wu et al. 2015). After electrophoresis, proteins in gels were visualized with CBB R350, and photographed using a DSLR camera (Nikon D7000) in automatic mode. The digital images of the gels were analyzed using PDQuest software (Bio-Rad, USA). The relative abundance of protein spots was estimated by taking the average normalized peak area of all spots in 2DE gels. The spots with a significant and consistent change in abundance in the replicate gels of the three biological replicates (Student’s t test, p < .05, fold-change ≥2.0) were regarded as the DAPs.

Mass spectrometry was performed as described previously (Niu et al. 2019). The spots of the DAPs were excised and subjected to in-gel trypsin digestion overnight. The digests fragments were subjected to MALDI-TOF/TOF analysis (ABI 5800, Applied Biosystems, CA, USA). The acquired spectra were automatically submitted to Mascot server (Matrix Science, London, UK) for protein identification against the UniProtKB (https://www.uniprot.org/) database (version 2016_08_16, 210842 sequences of Z. mays). Positive identification required significant scores (p < 0.05) and at least two unique matches. The identified DAPs were grouped by referring to the annotations in the UniProtKB database regarding cellular component, molecular function, and biological process. Subcellular locations were determined according to the annotation in UniProtKB database or predicted by homologous proteins or at Plant-mPLoc server (http://www.csbio.sjtu.edu.cn/bioinf/Plant multi/).

2.8 | Total RNA extraction and reverse transcription quantitative PCR (RT-qPCR) analysis

Total RNA of maize coleoptiles was extracted with RNA-Solv® reagent (Omega Bio-Tek, USA) according to the manufacturer’s instructions. The purity and concentration of total RNA were determined by a NanoDrop One spectrophotometer (Thermo Scientific, USA). First-strand cDNA synthesis was conducted using the 5xAll-In-One MasterMix (with AccuRT Genomic DNA Removal Kit) (Applied Biological Materials Inc., Richmond, Canada) according to the manufacturer’s instructions.

RT-qPCR was conducted in 96-well plates and performed on the StepOnePlus™ Real-Time PCR Instrument Thermal Cycling Block (Applied Bio-systems) under the cycling conditions (95°C for 30 s, followed by 40 cycles at 95°C for 10 s, 58°C for 10 s, and 72°C for 30 s). Each reaction was performed in a final volume of 20 μl containing...
10 μl SYBR Green Master Mix reagent (Bioman Technology Co., Ltd, Beijing, China), 200 ng of diluted cDNA sample, and 200 nM gene-specific primers. The gene-specific primers were designed using Primer 3.0 software and commercially synthesized (Biomed Cooperation, Beijing, China; Table S1). Amplification of the ZmUBI gene was used as an internal reference. The relative expression level of each gene was calculated by the $2^{-\Delta\Delta CT}$ method. Each sample was carried out at least three biological replicates. Statistically significant differences in changes in gene expression (|fold-change| ≥ 1.5) due to treatments were accessed by a Student’s t test (*p < .05, **p < .01 and ***p < .001).

2.9 Prediction of the phytohormone-related cis-acting motifs

The 1,000-bp promoter sequences of the genes encoding DAPs were retrieved from the maize genomic sequence available at NCBI (https://www.ncbi.nlm.nih.gov/). A search of the extracted promoter sequences for phytohormone-related cis-motifs was conducted using the Plant-CARE (Plant Cis-Acting Regulatory Element, https://bioinformatics.psb.ugent.be/webtools/plantcare/html) (Lescot et al. 2002). The distribution of phytohormone-responsive cis-acting motifs was plotted using Microsoft Excel.
3 | RESULTS

3.1 | Growth of etiolated coleoptile

Growth index of the coleoptiles of etiolated maize seedlings (Figure 1a) was assayed daily over 7 DAG. The changes in length and DW of the coleoptiles displayed characteristic S-shaped curves (Figure 1b), as reported previously (Tetley & Priestley, 1927). The coleoptiles reached the maximal DW and length at 4 and 5 DAG, respectively. Clearly, the coleoptiles elongated rapidly during 2–5 DAG, with an average growth rate of 750 μm/hr. Thus, the coleoptiles at 1.5, 3, and 5 DAG, representing lag phase, exponential phase, stationary phase, individually, were used for further analysis.

Microscopic observation indicated that the coleoptile was surrounded by an epidermis and within the epidermis, wall-thinned cells (parenchyma) with prominent nuclei were poorly differentiated (Figure 1c). The coleoptile cells became 5–6 times in length at 5 DAG than at 1.5 DAG and somewhat wider. Two opposite, simple vascular bundles were observed in the cross section of coleoptiles (Figure 1d). Thus, the rapid growth of the coleoptiles was due to longitudinal extension of coleoptile cells.

3.2 | Phytohormone changes with coleoptile growth

To examine the changes in the levels of major phytohormones and phytohormone-responsive DAPs, the contents of IAA, SA, JA, GA3, and ABA were quantified per FW in elongating coleoptiles (Figure 2). The contents of IAA, SA, and JA decreased significantly during the growth of coleoptile by 3.07, 3.21, and 8.06 times, respectively. GA3 first decreased and then increased. In the coleoptile at 3 DAG, the content of GA3 was only 1.11 ng/g FW. ABA showed no significant changes during the growth of the coleoptiles.

3.3 | Protein changes with coleoptile growth

Protein content in growing coleoptiles was assayed based on equal FW, DW, and equal number of coleoptile individuals. In the growing coleoptiles, protein content decreased per FW and DW, but increased in individual coleoptile (Figure 3a). This difference could be partly explained by growth characteristics of coleoptiles: DW reached the maximum at 4 DAG, whereas the length at 5 DAG (Figure 1b), that is, the growth of coleoptiles was mainly driven by the increased water input and wall growth.

The quantitative protein changes in the growing coleoptiles were further detected using 2DE at 1.5, 3, and 5 DAG (Figure 3b, Figure S1). PDQuest comparison of 2DE images revealed that among approximately 700 protein spots of each gel, 44 were identified as the DAPs with significant and reproducible changes (Figure S2; Student’s t test, p < .05). These DAPs were grouped as three types (Table 1): I, with steadily increased abundance (24 spots); II, with the highest abundance at 3 DAG (6 spots); III, with gradually decreased abundance (14 spots).

3.4 | DAPs associated with coleoptile growth

The 44 DAPs were successfully identified by mass spectrometry, belonging to 31 different kinds of proteins (Table 1, Table S2).
According to the annotation in the UniProtKB, these proteins take part in various cellular processes. More importantly, the DAPs of types I and II were involved in growth-related cellular processes, e.g., translational initiation, transcription regulation, protein biosynthesis, aromatic compound/lignin biosynthesis, H+ transmembrane transport, and cytoskeleton organization. Notably, the DAPs involved in stress response, especially herbicide safener binding protein (SBP), ABA stress ripening 2, POD, glutathione S-transferase 3, and kiwellin were highly accumulated with coleoptile growth. In particular, several DAPs increased from undetected levels to high levels, such as actin (spot 29), O-methyltransferase ZRP4 (spot 39), SBP1 (spots 38, 40, 41), ABA stress ripening 2 (spot 44), profilin-5 (spot 36), hydroxyproline-rich glycoprotein (spot 42), and legumin-like protein (spot 43). In type III, the DAPs with nutrient reservoir activities (embryonic protein DC-8, globulin-2, oil body-associated protein 2B) were degraded to provide energy for coleoptile growth. Therefore, the cellular processes, especially growth- and stress-related processes, were selectively enhanced to support the needs of the rapidly growing coleoptiles.

### 3.5 Verification of the DAPs

The activities of soluble POD and wall-bound POD increased significantly with the elongation of coleoptile, and the activity of wall-bound POD was higher than that of soluble POD (Figure 4a). Soluble POD activity staining showed that isozyme patterns significantly changed with coleoptile elongation, especially two major POD activities emerged (Figure 4b). These results were largely consistent with POD abundance changes in 2DE images (Figure S2, spots 19, 31 and 32) during germination.

Immunoblot analysis showed that on an equal protein basis, the accumulation of actin, tubulin, and expansin strongly increased with coleoptile growth, with the highest abundance at 5 DAG (Figure S3a,b); nevertheless, for a single coleoptile, they accumulated to the highest abundance at 3 DAG (Figure S3c,d). These results were consistent with the results of 2DE.

RT-qPCR showed that the expression patterns (Figure 5) of 11 selected genes in the growing coleoptiles were partly consistent with the abundance changes (Table 1) of the corresponding DAPs.
| DAPs type | Protein, spot | UniProtKB accession | Cellular component | Molecular function | Biological process |
|-----------|--------------|---------------------|-------------------|-------------------|-------------------|
| Type I with steadily increased abundance from 1.5 to 5 DAG | β-D-Glucoside glucohydrolase, spots 1-3 | P49235 | Plastid | β-Glucosidase activity | Carbohydrate metabolism |
| | Nucleoside diphosphate kinase 1, spot 27 | B4FK49 | Nucleus | DNA, ATP binding | Transcription regulation |
| | Glutathione S-transferase 3, spot 33 | B4FTD1 | Cytoplasm | Transferase activity | Glutathione metabolism, response to herbicide |
| | O-methyltransferase ZRP4, spot 39 | B6TS22 | Plastid | Methyltransferase | Aromatic compound biosynthesis |
| | S-adenosylmethionine synthase, spot 35 | K7VC35 | Cytosol | Methionine adenosyltransferase activity | S-adenosylmethionine biosynthesis |
| | H^+ -ATPase subunit B, spot 5 | B6T9C0 | Vacuolar tonoplast | ATP binding | H^+ transmembrane transport |
| | Eukaryotic initiation factor 4a (eIF4A), spot 6 | A0A0B44J303 | Cytosol, nucleus | RNA helicase activity | Translational initiation, protein biosynthesis |
| | Ascorbate peroxidase 1, spots 19, 32 | B6U9S6, B6TM55 | Cytosol, peroxisome | L-ascorbate peroxidase activity | Response to oxidative stress |
| | Ascorbate peroxidase 2, spot 31 | B4G031 | Plastid (chloroplast) | L-ascorbate peroxidase activity | Response to oxidative stress |
| | Herbside safener binding protein (SBP1), spots 38, 40, 41 | Q49010 | Plastid | O-methyltransferase, protein dimerization activity | Aromatic compound, lignin biosynthesis, response to herbicide |
| | ABA stress ripening 2, spot 44 | B4G0Q9 | Nucleus, cytoplasm | Sequence-specific DNA binding | Transcription regulation, stress response |
| | Ferritin 1, spot 30 | P29036 | Plastid | Iron ion binding, ferroxidase activity | Iron ion storage and transport |
| | Profilin 5, spot 36 | Q9FR39 | Cytoskeleton | Actin binding | Regulation of cytoskeleton organization |
| | Kiwellin 1, spot 37 | A0A1D6GNR3 | Apoplast | Specifically blocks the enzyme Cmu1 from Ustilago maydis | Defense response to fungus |
| | Hydroxyproline-rich glycoprotein, spot 42 | B4G019 | Cell wall, secreted | Carbohydrate binding | Strengthening of walls |
| | Actin 7, spots 7, 8, 28, 29 | A0A1D6LJ44, B6TQ08, B4F989, C0HDZ6 | Cytoskeleton | Major constituent of microtubules | Cytoskeleton-based process, response to various stimuli |

(Continues)
| DAPs type                      | Protein, spot                                      | UniProtKB accession | Cellular component | Molecular function                                      | Biological process                                        |
|-------------------------------|---------------------------------------------------|---------------------|--------------------|---------------------------------------------------------|----------------------------------------------------------|
| **Type II**                   |                                                   |                     |                    |                                                         |                                                          |
| with maximum abundance        |                                                   |                     |                    |                                                         |                                                          |
| at 3 DAG                      |                                                   |                     |                    |                                                         |                                                          |
| Tubulin α-1 chain (TUBA1),    |                                                   | P14640              | Cytoskeleton       | Major constituent of microtubules                       | Microtubule-based process, cytoskeleton organization     |
| spot 4                        |                                                   |                     |                    |                                                         |                                                          |
| General regulatory factor 2,  |                                                   | K7TL05              | Nucleus            | Transcription factor, protein domain specific binding   | Transcription regulation                                  |
| spot 17                       |                                                   |                     |                    |                                                         |                                                          |
| Fructokinase-2 (FRK2), spot   |                                                   | Q6XZ78              | Cytosol            | Carbohydrate kinase activity                            | Carbohydrate metabolism (starch biosynthetic process)    |
| 9                            |                                                   |                     |                    |                                                         |                                                          |
| 14-3-3-like protein, spot 18  |                                                   | B6U284              | Nucleus            | DNA/protein domain specific binding                     | Transcription regulation                                  |
|                              |                                                   |                     |                    |                                                         |                                                          |
| Legumin-like protein, spot 43 |                                                   | Q84TL7              | Vacuole <sup>a</sup> | Nutrient reservoir activity                             | Seed maturation, germination                              |
|                              |                                                   |                     |                    |                                                         |                                                          |
| Major latex protein 22 (MLP 22, spot 34) |           | Q19VG6              | Vacuole, cytoplasmic | ABA binding, signaling receptor activity                | Defense response, ABA-activated signaling pathway          |
| **Type III**                  |                                                   |                     |                    |                                                         |                                                          |
| with gradually decreased      |                                                   |                     |                    |                                                         |                                                          |
| abundance from 1.5 to 5 DAG   |                                                   |                     |                    |                                                         |                                                          |
| NAD(P)-binding Rossmann-fold  |                                                   | A0A1D6F0W7          | Cytosol, plasma    | Oxidoreductase activity                                 | Defense response                                         |
| protein, spots 10, 12         |                                                   |                     | membrane           |                                                         |                                                          |
| Fructose-bisphosphate aldolase, spot 16 |               | P08440              | Cytoplasm           | Fructose-bisphosphate aldolase activity                | Glycolysis, sucrose biosynthetic process, response to anoxia |
| Guanine nucleotide-binding protein β subunit, spot 13 | | B6SJ21 | Cytosol, nucleus | Protein kinase C, ribosome binding | Positive regulation of protein phosphorylation, rescue of stalled ribosome |
| Embryonic protein DC-8, spot 11 |                          | A0A1D6JQ00          | Apoplast, cytoplasm | Late embryogeny                                       | Seed maturation, germination                              |
| Globulin-2, spots 14, 21, 22  |                                                   | Q7M1Z8              | Cell wall, vacuole | Nutrient reservoir activity                             | Seed maturation, germination                              |
| Oil body-associated protein 2B, spot 15 |                     | B6UI56              | Lipid droplet      | Lipid storage                                          | Seed maturation and germination                            |
| 1-Cys peroxiredoxin PER1, spot 20 |                          | A25ZW8              | Nucleus, cytoplasm  | Antioxidant, POD activity                              | Cell redox homeostasis, defense response to fungus        |
| 22.0 kDa heat shock protein, spots 23, 24 |            | C0PB6J4             | Endoplasmic reticulum | Chaperone, unfolded protein binding                   | Protein folding, stress response heat, reactive oxygen species and saline |
| 16.9 kDa class I heat shock protein, spots 25, 26 |                | B6SIX0              | Cytoplasm          | Chaperone, unfolded protein binding                     | Protein folding, response to various stimuli               |

<sup>a</sup>Localization prediction with online Plant-mPLoc sever.
For example, the expression of O-Methyltransferase ZRP4 steadily increased with coleoptile growth, as its product O-methyltransferase ZRP4 substantially accumulated; the levels of 16.9 kDa class I heat shock protein 1 and its gene sharply decreased with coleoptile growth, and so did Auxin-binding protein 1. However, several genes were inconsistent between gene expression and protein accumulation, such as Actin-7, Tubulin α-1 chain, Profilin-5.

Additionally, several hormone-responsive cis-acting elements were detected in the promoter sequences of these genes encoding DAPs, including the ABA-responsive element (ABRE), ethylene-responsive element (ERE), GA-responsive element (TATC-box/GARE-motif/P-box), auxin-responsive element (AuxRR-core/TGA-element), SA-responsive element (TCA-element), and MeJA responsive element (TGACG-motif/CGTCA-motif) (Table 2, Figure S4). Except 14–3–3-like protein, all DAPs contained at least one hormone-responsive cis-acting element; and several DAPs, e.g., actin-7, kiwellin-1, and fructokinase-2 held 5–6 hormone-responsive cis-acting elements.

4 | DISCUSSION

The coleoptile is a short-lived organ with low differentiation; its growth is highly dependent upon the elongation of the cells performed in the embryo (Jones & Rost, 1989). In the present study, we aimed to characterize proteome changes and the DAPs in the rapidly growing coleoptiles of maize. Due to space limitation, the discussion below focused on a few DAPs.

Our work showed the significant changes in protein content and 1D profiles with maize coleoptile growth, especially protein content per coleoptile steadily increased. 2DE analysis further revealed quantitative the proteome changes with 31 DAPs (44 spots) associated with rapid growth of the coleoptile. Therefore, the synthesis and degradation of specific proteins were selectively enhanced during coleoptile growth. Importantly, 21 DAPs with increased abundance (type I and II) are mainly involved in growth-related, and thus are necessary for rapid growth of the coleoptile. This implied that protein synthesis plays a vital role in rapid growth of maize coleoptile, as found in rice (Edwards et al. 2012; Lasanthi-Kudahettige et al. 2007; Narasai et al. 2009). For the first time, β-D-glucoside glucosylase, nucleoside diphosphate kinase1, ferritin-1, kiwellin-1, and ABA stress ripening 2 were implied to be involved in coleoptile growth.

Cell wall and cuticle play a key role in plant growth and form an outermost barrier against pathogen infection (Keegstra, 2010). Using maize coleoptile segments incubated in vitro, two proteins involved in cutin or cell wall biosynthesis were identified (Li et al. 2013). In the present study, the DAPs involved in lignin biosynthesis, aromatic compound biosynthesis, and strengthening of walls were highly accumulated with coleoptile growth. To grow, plants must loosen rigid walls in a pH-dependent manner (Arsuffi & Braybrook, 2018) by expansins (Choi et al. 2003; Cosgrove, 2000). Though expansins were not found as DAPs here possibly due to their low abundance, immunoblotting and RT-qPCR showed their change with coleoptile growth (Figure 5, Figure S3). POD plays a key role in H₂O₂-mediated cell wall stiffening in maize coleoptiles (Schopfer, 1996). In the present study, the increase in POD abundance and activity with coleoptile growth was consistent with the presence of POD in the growth-limiting outer epidermal wall (Schopfer, 1996), implying their role in cell wall stiffening and stress response.

Actin and tubulin are essential components of cell cytoskeleton, involved in cytoskeleton-based processes, e.g., cell division and elongation, vesicle transport, signal transduction, and cell wall deposition (Hashimoto, 2015; Kandasamy et al. 2001). The accumulation of α-tubulin, especially acetylated ones, gradually increased during
**FIGURE 5** Verification of transcript abundance of the genes encoding the selected DAPs. RT-qPCR was repeated in three biological replicates, with ZmUBI as a reference gene. Gene name in red indicates an inconsistency between gene expression and protein accumulation. Asterisks show significant differences in expression changes as assessed by a Student's t test (|fold-change| ≥ 1.5; *p < .05, **p < .01, ***p < .001).

**TABLE 2** Analysis of hormone-responsive cis-acting element in the promoter regions in the genes encoding DAPs

| Cis-acting element | Function                     | Sequence                               | Number of DAPs involved |
|--------------------|------------------------------|----------------------------------------|-------------------------|
| ABRE               | ABA responsive element       | CGTACGTGC or ACGTG or GACACGTACGT     | 22                      |
| ERE                | ethylene responsive element  | ATTTCAA                                | 4                       |
| TATC-box           | GA responsive element        | TATCCCA                                | 1                       |
| GARE-motif         | GA responsive element        | AAACAGA or TCTGTGT                    | 6                       |
| P-box              | GA responsive element        | GCCTTTTGAGT                            | 6                       |
| AuxRR-core         | auxin responsive element     | GGTCCAT                                | 4                       |
| TGA-element        | auxin responsive element     | ACGGAC                                 | 9                       |
| TCA-element        | SA responsive element        | CAGAAAGGA or GAGAAGAATA                | 7                       |
| TGACG-motif        | MeJA responsive element      | TGACG                                  | 16                      |
| CGTCA-motif        | MeJA responsive element      | CGTCA                                  | 16                      |
the early growth stages of *Brassica rapa* seedlings and correlated to the maturation of plant organ (Nakagawa et al. 2013). A similar situation existed with actin (Nakagawa et al. 2013). We here confirmed the increased expression of tubulin and actin by 2DE, immunoblotting and RT-qPCR, strongly showing their important roles in coleoptile growth.

In the present work, the mRNA levels of eleven DAPs were partly correlated with protein abundance (Figure 5). The discordance of transcript/protein level has been well documented in maize (Ponnala et al. 2014), possibly because mRNA is not a direct indication of protein level. The protein abundance can not only be regulated at transcription level but also translation level and turnover level.

The roles of auxin in rapid growth of maize coleoptile have been extensively studied before (Kutschera et al. 1987; Kutschera & Khanna, 2020). In the present study, 2DE analysis failed to identify any auxin-related proteins as the DAPs, this was mainly due to the possibility that 2-fold changes in abundance were set to filter DAPs. In the growing coleoptiles, the abundance changes of auxin-related proteins were possibly lower than twofolds based on equal protein amounts, or auxin-related proteins accumulated in low abundance, which could not be detected by 2DE combining CBB R350 staining due to the poor resolution. In a previous study, auxin-associated proteins were also not identified in IAA-induced rapidly growing maize coleoptiles by 2DE and mass spectrometry (Li et al. 2013). Thus, we performed experiments to assay auxin content and detect the gene expression of auxin-related proteins in growing maize coleoptile. Results found that the content of IAA decreased during coleoptile growth, coinciding with the expression of auxin-binding protein 1 (Figures 2 and 5). In addition, bioinformatic analysis showed that the promoter sequences of 12 DAPs-encoding genes (e.g., *Profilin-5, Actin-7, Kiwelin-1*, and *General regulatory factor 2*) contained auxin-responsive elements (*AuxRR-core/TGA-element*) (Table 2, Figure S4), suggesting these DAPs were regulated by auxin.

Peroxidases are a group of enzymes that catalyze the oxidation of a substrate by hydrogen peroxide or an organic peroxide. As discussed previously, antioxidant enzymes (e.g., peroxidases and superoxide dismutase) and their activities did not always match the extent of alteration in their abundance (Niu, Xu, et al. 2018). It is difficult to correlate specific enzyme activities to protein abundance. Soluble POD activity staining showed that isozyme patterns significantly changed with coleoptile elongation, especially two major POD activities emerged (Figure 4b). These results were largely consistent with POD abundance changes in 2DE images (Figure 52, spots 19, 31 and 32). These POD (spots 19, 31, and 32) were localized within cell internals rather in cell walls (Table 1). Thus, we inferred that the two major POD activities in Figure 4b possibly corresponded to spots 19, 31, and 32. However, the verification of POD identities in Figure 4b should cut the gel bands with POD activities and then subjected to mass spectrometry.

Our results showed that to respond to stress conditions during germination, coleoptiles substantially accumulated stress-responsive DAPs, especially herbicide SBP, ABA stress ripening 2, major latex protein 22, kiwelin, and NAD(P)-binding Rossmann-fold superfamily (Han et al. 2019; Hatzios, 1983; Yamauchi et al. 2011). Herbicide safeners have long been used to protect crops from herbicide injury (Hatzios, 1983). Cereal coleoptile is an important site for the action of herbicides and safeners (Scott-Craig et al. 1998). We found here that the abundance of SBP (spots 40 and 41) increased at least ten-thousand times at 3–5 DAG and became one of the most abundant proteins in the growing coleoptiles, suggesting that the coleoptiles have prepared themselves for confronting possible herbicide injury during germination.

It is worth noting that several enzymes identified here, especially H⁺-ATPase subunit B and β-glucosidase and fructokinase-2 were highly accumulated in the rapidly growing coleoptiles. The expression of H⁺-ATPase genes was found to positively correlate with cellular growth (Viereck et al. 1996). β-Glucosidase was found to be most abundant in the coleoptiles (Esen, 1992) and involved in carbohydrate metabolic process and cytokinin-activated signaling pathway. Fructokinase-2 takes part in starch biosynthesis and may also be involved in a sugar-sensing pathway (Zhang et al. 2003). The high abundance of these enzymes revealed here suggested their important roles in rapid growth of maize coleoptiles.

In addition, our work provides complementary results compared with the previous study (Li et al. 2013) that used SDS–PAGE and mass spectrometry to identify the proteins of the cultures in vitro of excised maize coleoptiles. Both studies indicated that eukaryotic initiation factor 4a (eIF4A), S-adenosylmethionine synthase, tubulin, SBP, 14-3-3-like protein, POD and profilin-5 increased in abundance either in vivo or in vitro growth of maize coleoptiles.

## 5 | CONCLUSION

Our results showed that the rapid growth of maize coleoptile is largely due to its ability to quickly enhance relevant cellular processes, especially the selective synthesis of the DAPs that are involved in growth-related cellular processes. This study will contribute to the understanding of the mechanisms underlying coleoptile growth in maize and other cereal plants. The DAPs identified here may be useful for regulating plant growth and for increased biomass in agriculture.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHORS’ CONTRIBUTIONS

W.W. conceived research; L.J.N., W.L.H., L.Y.L., and C.H.X. performed experiments and data analyses. X.L.W. analyzed data. L.J.N. drafted manuscript. X.L.W. and W.W. edited manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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