Physiological and comparative proteomic analyses provide insight into the differential responses of *Acanthus ilicifolius* and its relative, *Acanthus mollis*, to tidal flooding stress

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**Abstract:** The mangrove plant *Acanthus ilicifolius* and its relative, *A. mollis*, have been previously proved to possess diverse pharmacological effects. Therefore, evaluating the differentially expressed proteins of these species under tidal flooding stress is essential to fully exploit and benefit from their medicinal values. The roots of *A. ilicifolius* and *A. mollis* were exposed to 6 h of flooding stress per day for 10 days. The dry weight, hydrogen peroxide (H₂O₂) content, anatomical characteristics, carbon and energy levels, and two-dimensional electrophoresis coupled with MALDI-TOF/TOF MS technology were used to reveal the divergent flooding resistant strategies. *A. ilicifolius* performed better under tidal flooding stress, which was reflected in the integrity of the morphological structure, more efficient use of carbon and energy, and a higher percentage of up-regulated proteins associated with carbon and energy metabolism. *A. mollis* could not survive in flooding conditions for a long time, as revealed by incomplete cell structures of the roots, less efficient use of carbon and energy, and a higher percentage of down-regulated proteins associated with carbon and energy metabolism. Energy provision and flux balance played a role in the flooding tolerance of *A. ilicifolius* and *A. mollis*.

**Keywords:** Acanthus species; flooding stress; physiological; comparative proteomics analyses; carbon and energy metabolism

1. Introduction

The physical characteristics of soil influence a variety of physiological and biochemical processes of plants. The leaves and roots of terrestrial plants absorb molecular oxygen from air and land, respectively [1]. Previous studies have shown that flooding stress is a widespread phenomenon that inhibits plant growth and production [2]. Continuous and heavy rainfall causes soil pores saturated with excess water, inducing oxygen deficiency in plant roots [1-2]. Meanwhile, the roots that are subjected to flooding stress may inhibit photosynthesis, including a decrease of photosynthetic electron transport chain and an increase in the level of reactive oxygen species (ROS) [3]. A shift of aerobic respiration to anaerobic respiration reduced the availability of the adenosine triphosphate (ATP) in plants [4] and increasing the content of ethanol [5].

Some plants have evolved morphological, physiological, and metabolic adaptation strategies to ensure survival under flooding stress [6-7]. For example, maize develops an extensive aerenchyma system to facilitate gas transport apart from adventitious roots [6]. Rice retains a gas-associated electron transport chain and an increase in the level of reactive oxygen species (ROS) [3]. A shift of aerobic respiration to anaerobic respiration reduced the availability of the adenosine triphosphate (ATP) in plants [4] and increasing the content of ethanol [5].

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A comparative study of species is one of the important methods to determine the mechanism of stress-resistant. The difference in fermentative enzymes and alanine aminotransferase activity resulted in different responses to energy deficiency between two soybean genotypes under flooding conditions [9]. A comparison of Alternanthera philoxeroides with Hemarthria altissima showed that plants could adapt to wetland habitats, in which water levels fluctuate, by maintaining the functionality of the photosynthetic apparatus [11]. Waterlogged Phalaris aquatica and Festuca arundinacea regained growth during the recovery period compared with Dactylis glomerata and Bromus catharticus [12]. In addition, the favorable alleles of related species are more comfortable to introduce to improve crops [13]. The transfer of resistance genes between Sinapis alba and Brassica species by somatic and sexual hybridization has been accomplished [14].

The mangrove plant, Acanthus ilicifolius, has remarkable morphology and physiology [15]. A. ilicifolius is mainly distributed in Australia, Australasia, and the southeastern Asia intertidal zone and has numerous medicinal properties [16]. Previous findings showed that untreated or submerged A. ilicifolius over 3 h per day was not conducive to the growth [17]. Meanwhile, the effect of flooding stress on A. ilicifolius at the molecular level is not well elucidated. Acanthus belongs to the Acanthaceae family and is the only genus that comprises of both terrestrial and aquatic species [15]. As the Acanthus model plant, A. mollis is native to the Mediterranean region from central Europe and northwest Africa [18]. A. mollis was recently introduced into China and used as a medicinal plant in traditional medicine [18-19]. The extracts of A. mollis tissues have been used for the treatment of inflammation and cancer problems [20]. However, the flooding tolerance of A. mollis has not yet been described. Since non-model plants lack a genetic transformation system to elucidate the metabolic mechanism, proteome and transcriptome are useful to provide powerful information about the metabolic pathways of non-model plants. The protein, as the functional executor, is closely related to physiological changes. In our previous study, we have reported the flooding tolerance of the leaves of Avicennia marina using comparative proteomic analyses [21]. Hence, evaluating the differentially expressed proteins (DEPs) of A. ilicifolius and A. mollis under tidal flooding stress is essential to fully exploit and benefit from their medicinal values.

Paraffin sections, physiological index measurements, and two-dimensional electrophoresis (2-DE) technique were performed on the leaves and roots of A. ilicifolius and A. mollis under tidal flooding stress. Our results first provide the anatomical characteristics, carbon and energy levels, and proteomic information about A. ilicifolius and its relative, A. mollis, under tidal flooding stress.

2. Results

2.1. Relative dry weight and H2O2 content of Acanthus species under tidal flooding stress

The two species changed the relative dry weight and H2O2 content to differing extents under tidal flooding stress. The relative dry weight of A. ilicifolius was increased in both the leaf and root tissues (Fig. 1A) but with no significant differences. The relative H2O2 content was significantly decreased on the tenth day in A. ilicifolius leaves and showed no significant differences from day 4-12 in A. ilicifolius roots (Fig. 1C). Tidal flooding treatment significantly decreased the relative dry weight (Fig. 1B) and increased the relative H2O2 content of A. mollis in both tissues (Fig. 1D). Overall, the relative dry weight (expressed as a percentage of the control) of A. ilicifolius was significantly higher compared to that of A. mollis from day 8-12.
2.2. Effect of tidal flooding on the phenotype and anatomical characteristics of Acanthus species

The phenotype and anatomical characteristics of the two species are shown in Fig. 2. After 10 days of tidal flooding treatment, there was no significant change in A. ilicifolius (Fig. 2A1, B1), while the length of A. mollis roots became shorter (Fig. 2C1, D1).

The leaf blade of A. ilicifolius consisted of the upper epidermis, upper multiple epidermises, palisade parenchyma, spongy parenchyma, lower epidermis, and salt gland (Fig. 2A2), whereas that of A. mollis showed a different structure. The upper and lower epidermises of A. mollis were all single-layered and had glandular trichome (Fig. 2C2). A. ilicifolius roots consisted of the endodermis, epidermis, xylem, phloem, pith, cortex, lenticel, and periderm (Fig. 2A4, A5) whereas the roots of A. mollis contained periderm cells (Fig. 2D5).

In the leaf blade of A. ilicifolius, the vein phloem possessed a hollow cavity that was enlarged after tidal flooding treatment (Fig. 2B3). The control group of A. mollis exhibited schizogenous aerenchyma in the leaf vein, which disappeared after tidal flooding treatment. The periderm is a secondary protective tissue that protects plant roots from bacterial infections [22]. In A. mollis roots, the pith parenchyma cells were damaged and the periderm cells were ruptured under tidal flooding stress (Fig. 2D4, D5).
Figure 2. Phenotypic and anatomical changes of A. ilicifolius and A. mollis exposed to tidal flooding stress. (A1-A5) A. ilicifolius plants on control treatment, (B1-B5) A. ilicifolius plants under tidal flooding stress, (C1-C5) A. mollis plants on control treatment, (D1-D5) A. mollis plants under tidal flooding stress. Row 1: the phenotypic of Acanthus species, Row 2: the cross-section of the leaf blade, Row 3: the main vein of the leaf, Row 4: stele of root, Row 5: epidermis of root. Root sections, about 5 cm from root tip, photos of optical microscopes. Cross-sections with thickness of 10 mm were made and stained with safranine and fast green. cc: cork cambium; co: collenchyma; ct: cortex; en: endodermis; Ep: epidermis; gt: glandular trichome; le: lenticel; pe: periderm; ph: phloem; pi: pith; pp: palisade parenchyma; sa: schizogenous aerenchyma; st: stomata; sp: lacunar parenchyma; xy: xylem. Bars: 100 μm.

2.3. Identification and quantification of tidal flooding-responsive proteins

Representative 2-DE gels of the leaves and roots of the two species are shown in Supplemental Fig. S3. In A. ilicifolius, approximately 78 and 40 spots were identified from leaves and roots, respectively (Fig. 3A, B; Table 1, 2; Supplementary Table S2). In A. mollis, about 67 and 45 spots were identified from leaves and roots, respectively (Fig. 3C, D; Table 3, 4; Supplementary Table S2).

To understand the global relationship between samples, PCA was performed to evaluate the similarity between the samples (Fig. 4). The weights of the first principal component (PC1) satisfied the cumulative percent variance (70–85%) [23], accounting for nearly 80% of the difference. The control group (CK) and the soil flooding group (SF) separated from each other. The excellent biological repeatability of these proteomes, derived from the same condition, indicates a stable and distinct response of A. ilicifolius and A. mollis to tidal flooding treatment.
Figure 3. Two-dimensional (2-DE) analysis of proteins extracted from (A) *A. ilicifolius* leaves, (B) *A. ilicifolius* roots, (C) *A. mollis* leaves, (D) *A. ilicifolius* roots. The numbers correspond with the spot ID, mentioned in Table 1-4. The isoelectric point (pI) and molecular weight (MW) in kilodaltons are indicated on the top and left of the gel, respectively. CK and SF represent the control group and soil tidal flooding stress, respectively.

2.4. **Functional classification of DEPs**

More proteins were up-regulated in *A. ilicifolius* than in *A. mollis* tissues (Fig. 5). A higher percentage of up-regulated proteins were found in carbon and energy metabolism and amino acid and protein metabolism in *A. mollis* leaves, while in transcription and signal transduction in *A. ilicifolius* leaves. In addition, *A. ilicifolius* leaves had a lower percentage of up-regulated proteins associated with stress and defense. Overall, *A. ilicifolius* tissues had a high percentage of up-regulated proteins and a low percentage of down-regulated proteins associated with carbon and energy metabolism. Meanwhile, a higher percentage of down-regulated proteins of *A. mollis* leaves were associated with carbon and energy metabolism, stress and defense, and transcription and signal transduction (Fig. 5).

Compared with *A. mollis* roots, a higher percentage of up-regulated proteins of *A. ilicifolius* roots were associated with carbon and energy metabolism, amino acid and protein metabolism, stress and defense, and transcription and signal transduction. In *A. mollis* roots, there was a higher percentage of down-regulated proteins in all pathways (Fig. 5).
Figure 4. Principal Component Analysis (PCA) of total proteome data for (A) *A. ilicifolius* leaves, (B) *A. ilicifolius* roots, (C) *A. mollis* leaves, (D) *A. mollis* roots. Percentage variance for each principal component is given.

Figure 5. Functional classification analysis of DEPs of *A. ilicifolius* and *A. mollis*. The detailed information for each spots is shown in Table 1-4.
### Table 1. Identification of DEPs of *A. ilicifolius* leaves with an expression change greater than 2.0-fold change under tidal flooding stress

| Spot | Accession (gb) | Protein Name                  | Thero. \(^d\) kDa/pI | Exper. \(^e\) kDa/pI | Score \(^f\) | MP | Species \(^b\) | SF vs. CK |
|------|----------------|-------------------------------|------------------------|-----------------------|--------------|----|----------------|-----------|
|      | gi|222842405 | Plastocyanin family protein | 17.07/4.94             | 6.80/4.00             | 98           | 2  | *Populus trichocarpa* | -6.268    |
|      | gi|449515811 | Predicted: chlorophyll a-b binding protein 40, chloroplast-like, partial | 15.98/6.58             | 18.87/4.93             | 88           | 2  | Cucumis sativus          | -1.555    |
|      | gi|222859802 | Chlorophyll a-b binding protein 2 | 28.09/5.29             | 15.44/5.07             | 87           | 6  | *Populus trichocarpa*      | 1.937     |
|      | gi|475542040 | Chlorophyll a-b binding protein, chloroplast | 28.72/5.14             | 19.47/4.74             | 114          | 6  | *Aegilops tauschii*         | 3.202     |
|      | gi|449442663 | Predicted: phosphoglycolate phosphatase-like | 41.72/6.47             | 24.00/4.78             | 63           | 3  | Cucumis sativus          | 3.455     |
|      | gi|474352688 | Oxygen-evolving enhancer protein 1, chloroplast | 34.64/5.75             | 36.97/4.93             | 352          | 7  | *Triticum urartu*           | 2.893     |
|      | gi|223540996 | Chlorophyll a/b binding protein, putative | 31.10/5.52             | 16.37/5.43             | 75           | 5  | *Ricinus communis*        | -1.887    |
|      | gi|428230860 | Chlorophyll binding protein, partial | 21.39/5.19             | 15.41/5.93             | 66           | 2  | Clermontia arborescens subsp. Waihiae | 6.846     |
|      | gi|475616276 | Putative quinone-oxidoreductase-like protein, chloroplast | 35.43/9.13             | 27.96/5.86             | 69           | 11 | *Aegilops tauschii*       | -2.415    |
|      | gi|550338673 | Chain A family protein | 40.79/8.54             | 23.76/6.45             | 70           | 10 | *Populus trichocarpa*      | -6.268    |
|      | gi|475522663 | Ribulose bisphosphate carboxylase/oxygenase activase B, chloroplast | 51.47/8.86             | 38.41/4.86             | 268          | 5  | *Aegilops tauschii*         | -5.046    |

\(^a\)Spot number

\(^b\)Accession number

\(^c\)Protein name

\(^d\)Thermostable

\(^e\)Experimental

\(^f\)Score
|    | gi   | Description                                                                 | Score 1 | Score 2 | Score 3 | Score 4 | Species                  | Score 5 |
|----|------|----------------------------------------------------------------------------|---------|---------|---------|---------|--------------------------|---------|
| 5  | gi508726181 | Rubisco activase isoform 2                                                 | 52.37/5.26 | 37.73/4.91 | 407     | 10      | Theobroma cacao          | 1.103   |
| 7  | gi508787184 | RuBisCO large subunit-binding protein subunit alpha isoform 1              | 64.07/5.06 | 58.37/4.67 | 108     | 9       | Theobroma cacao          | 1.518   |
| 9  | gi502125499 | Predicted: RuBisCO large subunit-binding protein subunit beta, chloroplast-like | 63.20/5.85 | 52.72/5.05 | 162     | 6       | Cicer arietinum          | 5.431   |
| 15 | gi542718032 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) | 38.09/6.49 | 25.25/5.22 | 294     | 11      | Prunus wilsoni           | 3.362   |
| 19 | gi410927414 | Chloroplast ribulose bisphosphate carboxylase/oxygenase activase beta1, partial (chloroplast) | 33.16/5.09 | 32.55/5.14 | 274     | 4       | Gossypium barbadense     | 1.568   |
| 39 | gi223541989 | Phosphoribulose kinase, putative                                            | 45.22/5.83 | 30.46/5.31 | 302     | 11      | Ricinus communis         | 2.349   |
| 51 | gi399139809 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial      | 51.58/6.30 | 20.22/6.70 | 388     | 9       | Strobilanthes glutinosus | -3.465  |
| 61 | gi406366546 | Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, partial (chloroplast) | 19.30/8.80 | 23.30/6.17 | 61      | 8       | Gossypium gossypioides   | 1.000   |
| 34 | gi449458564 | Predicted: triosephosphate isomerase, chloroplast-like                      | 33.00/7.01 | 17.22/5.56 | 81      | 4       | Cucumis sativus          | 1.349   |
| 38 | gi131737397 | Chloroplast phosphoglycerate kinase 3                                     | 50.28/6.69 | 32.06/5.55 | 121     | 10      | Helianthus annuus        | 1.671   |
| 46 | gi527186354 | Triosephosphate isomerase                                                  | 33.67/6.90 | 16.94/5.83 | 314     | 6       | Gentianopsis aurea       | 3.564   |
| 48 | gi332005925 | 6-phosphogluconolactonase                                                  | 29.51/6.23 | 17.67/5.40 | 91      | 2       | Arabidopsis thaliana     | 2.677   |
| 49 | gi222864107 | Cytosolic phosphoglycerate kinase family protein                            | 42.77/5.70 | 30.36/5.89 | 314     | 7       | Populus trichocarpa      | 2.056   |

Carbon metabolism
| #  | gi/| Accession Number | Description | Expression | Expression | Species | Expression |
|----|-----|-------------------|-------------|------------|------------|---------|------------|
| 52 | gi|223547261 | Phosphoglycerate kinase, putative | 50.11/8.74 | 35.17/5.64 | 95 2 | Ricinus communis |
| 56 | gi|350538295 | Enolase | 48.05/5.68 | 46.18/5.87 | 233 8 | Solanum lycopersicum |
| 68 | gi|485820030 | Enolase, partial | 11.86/7.98 | 41.86/5.95 | 84 3 | Schiedea helleri |
| 73 | gi|508711124 | Glyceraldehyde-3-phosphate dehydrogenase A subunit2 | 43.26/8.15 | 35.69/6.73 | 407 11 | Theobroma cacao |
| 75 | gi|413916139 | Glycine cleavage complex P-protein | 119.11/6.79 | 88.83/6.36 | 118 8 | Zea mays |
| 76 | gi|413916139 | Glycine cleavage complex P-protein | 119.11/6.79 | 89.61/6.42 | 99 8 | Zea mays |

**TCA cycle**

| #  | gi/| Accession Number | Description | Expression | Expression | Species | Expression |
|----|-----|-------------------|-------------|------------|------------|---------|------------|
| 63 | gi|332189573 | Malate dehydrogenase | 35.89/6.11 | 28.43/5.94 | 70 7 | Arabidopsis thaliana |
| 64 | gi|223526678 | Malate dehydrogenase, putative | 35.98/6.40 | 28.39/6.19 | 183 2 | Ricinus communis |
| 74 | gi|499138229 | Dihydrolipoamide dehydrogenase, partial | 43.94/7.79 | 50.17/6.64 | 145 4 | Rhizophora stylosa |

**Energy metabolism**

| #  | gi/| Accession Number | Description | Expression | Expression | Species | Expression |
|----|-----|-------------------|-------------|------------|------------|---------|------------|
| 6  | gi|449454235 | Predicted: V-type proton ATPase subunit B 1-like | 54.36/4.96 | 47.50/4.88 | 103 10 | Cucumis sativus |
| 21 | gi|545719412 | ATP synthase CF1 alpha subunit (chloroplast) | 55.57/5.15 | 48.13/5.10 | 547 18 | Allosyncarpia ternata |
| 23 | gi|393396089 | ATP synthase CF1 beta subunit (chloroplast) | 53.80/5.20 | 44.61/5.25 | 774 18 | Vigna unguiculata |
| 24 | gi|449434570 | Predicted: ATP synthase subunit beta, mitochondrial-like | 59.89/5.90 | 45.30/5.29 | 571 16 | Cucumis sativus |
| 32 | gi|508726652 | ATP synthase D chain, mitochondrial | 19.65/0.00 | 11.60/5.35 | 98 5 | Theobroma cacao |
| 43 | gi|350537279 | Vacuolar H^+-ATPase A2 subunit isoform | 68.96/5.30 | 58.53/5.43 | 534 19 | Solanum lycopersicum |
| 44 | gi|350537129 | Vacuolar H^+-ATPase A1 subunit | 68.81/5.20 | 60.95/5.35 | 268 14 | Solanum lycopersicum |

**TCA cycle**

| #  | gi/| Accession Number | Description | Expression | Expression | Species | Expression |
|----|-----|-------------------|-------------|------------|------------|---------|------------|
| 52 | gi|223547261 | Phosphoglycerate kinase, putative | 50.11/8.74 | 35.17/5.64 | 95 2 | Ricinus communis |
| 56 | gi|350538295 | Enolase | 48.05/5.68 | 46.18/5.87 | 233 8 | Solanum lycopersicum |
| 68 | gi|485820030 | Enolase, partial | 11.86/7.98 | 41.86/5.95 | 84 3 | Schiedea helleri |
| 73 | gi|508711124 | Glyceraldehyde-3-phosphate dehydrogenase A subunit2 | 43.26/8.15 | 35.69/6.73 | 407 11 | Theobroma cacao |
| 75 | gi|413916139 | Glycine cleavage complex P-protein | 119.11/6.79 | 88.83/6.36 | 118 8 | Zea mays |
| 76 | gi|413916139 | Glycine cleavage complex P-protein | 119.11/6.79 | 89.61/6.42 | 99 8 | Zea mays |

**Energy metabolism**

| #  | gi/| Accession Number | Description | Expression | Expression | Species | Expression |
|----|-----|-------------------|-------------|------------|------------|---------|------------|
| 6  | gi|449454235 | Predicted: V-type proton ATPase subunit B 1-like | 54.36/4.96 | 47.50/4.88 | 103 10 | Cucumis sativus |
| 21 | gi|545719412 | ATP synthase CF1 alpha subunit (chloroplast) | 55.57/5.15 | 48.13/5.10 | 547 18 | Allosyncarpia ternata |
| 23 | gi|393396089 | ATP synthase CF1 beta subunit (chloroplast) | 53.80/5.20 | 44.61/5.25 | 774 18 | Vigna unguiculata |
| 24 | gi|449434570 | Predicted: ATP synthase subunit beta, mitochondrial-like | 59.89/5.90 | 45.30/5.29 | 571 16 | Cucumis sativus |
| 32 | gi|508726652 | ATP synthase D chain, mitochondrial | 19.65/0.00 | 11.60/5.35 | 98 5 | Theobroma cacao |
| 43 | gi|350537279 | Vacuolar H^+-ATPase A2 subunit isoform | 68.96/5.30 | 58.53/5.43 | 534 19 | Solanum lycopersicum |
| 44 | gi|350537129 | Vacuolar H^+-ATPase A1 subunit | 68.81/5.20 | 60.95/5.35 | 268 14 | Solanum lycopersicum |
|   | gi | isoform                                                                 | lycopersicum          | Ricinus communis | Populus trichocarpa | 1.578 | 1.438 |
|---|----|-------------------------------------------------------------------------|-----------------------|------------------|---------------------|-------|-------|
| 45| 223550217 | ATP-dependent clp protease, putative                                  |                      |                   |                     |       |
| 50| 222848536 | ATP synthase gamma chain 1 family protein                              |                      |                   |                     |       |
| 8  | 449442347 | Predicted: stromal 70 kDa heat shock-related protein, chloroplastic-like |                      |                   |                     |       |
| 10 | 332003097 | Heat shock cognate protein 70-1                                       |                      |                   |                     |       |
| 11 | 460369188 | Predicted: stromal 70 kDa heat shock-related protein, chloroplastic-like |                      |                   |                     |       |
| 17 | 508722909 | 20S proteasome alpha subunit F2                                        |                      |                   |                     |       |
| 25 | 508784980 | TCP-1/cpn60 chaperonin family protein                                   |                      |                   |                     |       |
| 26 | 508726275 | Heat shock cognate protein 70-1                                        |                      |                   |                     |       |
| 27 | 508784980 | TCP-1/cpn60 chaperonin family protein                                   |                      |                   |                     |       |
| 28 | 293334615 | Heat shock cognate 70 kDa protein 2                                    |                      |                   |                     |       |
| 29 | 332643321 | Heat shock protein 60                                                  |                      |                   |                     |       |
| 35 | 223544718 | Groes chaperonin, putative                                             |                      |                   |                     |       |
| 37 | 425856442 | Mta/sah nucleosidase, partial                                          |                      |                   |                     |       |
| 53 | 343465772 | Plastid glutamine synthetase isoform                                   |                      |                   |                     |       |

Amino acid and protein metabolism

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 December 2020 doi:10.20944/preprints202012.0169.v1
|   | gi   | Description                                                                 | Triticum durum | Arabidopsis thaliana | Aegilops tauschii | Aegilops tauschii | Arabidopsis thaliana | Populus trichocarpa | Zea mays | Phaseolus vulgaris | Stress and defense | Transcription and signal transduction |
|---|------|-----------------------------------------------------------------------------|----------------|---------------------|------------------|------------------|---------------------|---------------------|----------|--------------------|-------------------|--------------------------------------|
|54 | gi32642427 | S-adenosyethionine synthase 4                                              | 43.17/5.51     | 37.04/5.81          | 193              | 8                | Triticum urartu     | 1.505               | -1.269  | -1.781             | -2.900            | -1.714 |
|55 | gi475453557 | 26S protease regulatory subunit 6B-like protein                           | 30.13/6.54     | 46.00/5.57          | 155              | 9                | Litchi chinensis   | -6.172              | 1.505               | 0                   | 2.365 |
|57 | gi475603792 | Heat shock 70 kDa protein, mitochondrial                                    | 72.91/5.53     | 58.32/5.58          | 113              | 5                | Solanum lycopersicum | 2.365               | 1.505               | 2.265            | 1.661 |
|59 | gi332646593 | Proteasome subunit beta type-1                                             | 24.86/6.95     | 16.99/6.04          | 149              | 4                | Cucumis sativus     | -5.867               | 1.505               | 1.970            | 1.890 |
|66 | gi550319185 | Glutamate-ammonia ligase family protein                                    | 39.42/5.95     | 32.83/6.07          | 153              | 7                | Triticum urartu     | 1.505               |                     |                   |                     |
|67 | gi332642304 | Mitochondrial processing peptidase alpha subunit                          | 54.19/6.04     | 39.89/6.12          | 104              | 2                | Litchi chinensis   | -6.172              | 1.505               | 2.365            | 1.661 |
|69 | gi449450860 | Elongation factor 2-like                                                   | 95.03/5.97     | 86.42/6.25          | 133              | 8                | Cucumis sativus     | -3.222               | 1.505               | 2.365            | 1.661 |
|77 | gi226491656 | Peptidyl-prolyl cis-trans isomerase                                        | 26.37/0.00     | 10.97/6.84          | 155              | 6                | Theobroma cacao    | 1.983                | 1.505               | 2.365            | 1.661 |
|78 | gi543177006 | Peptidyl-prolyl cis-trans isomerase                                        | 27.37/9.46     | 17.33/6.99          | 277              | 4                | Theobroma cacao    | 1.983                | 1.505               | 2.365            | 1.661 |

Stress and defense

|   | gi   | Description                                                                 | Triticum durum | Arabidopsis thaliana | Aegilops tauschii | Aegilops tauschii |
|---|------|-----------------------------------------------------------------------------|----------------|---------------------|------------------|------------------|
|36 | gi474311703 | L-ascorbate peroxidase 1, cytosolic                                          | 27.56/5.85     | 18.92/5.45          | 117              | 5                |
|70 | gi436805717 | Copper/zinc-superoxide dismutase                                            | 15.39/5.47     | 8.88/6.22           | 96               | 2                |

Transcription and signal transduction

|   | gi   | Description                                                                 | Triticum durum | Arabidopsis thaliana | Aegilops tauschii | Aegilops tauschii |
|---|------|-----------------------------------------------------------------------------|----------------|---------------------|------------------|------------------|
|3  | gi26454609 | 14-3-3 protein 7                                                           | 28.91/4.96     | 22.71/4.65          | 137              | 3                |
|14  | gi449469841 | Predicted: 14-3-3-like protein-like                                          | 29.64/4.77     | 19.79/4.67          | 90               | 3                |
|22  | gi508718683 | Tubulin alpha-5                                                            | 54.00/4.98     | 44.65/5.20          | 433              | 10               |
|30  | gi449464210 | Predicted: leukotriene A-4 hydrolase homolog                                | 69.93/5.37     | 67.72/4.88          | 157              | 5                |
| No. | Accession | Description | Fold Change | p-Value | Score | Organism | Score |
|-----|-----------|-------------|-------------|---------|-------|----------|-------|
| 31  | gi|508782306 | Eukaryotic translation initiation factor 5A-1 | 17.77/5.60 | 115   | 3 | Theobroma cacao | 2.854 |
| 40  | gi|386278562 | Actin7a, partial | 39.39/5.21 | 271   | 9 | Vernicia fordii | 2.976 |
| 41  | gi|223540420 | Cell division protein ftsH, putative | 75.50/6.43 | 467   | 7 | Ricinus communis | -1.196 |
| 42  | gi|475605012 | Cell division protease ftsH-like protein, chloroplastic | 71.94/5.60 | 271   | 8 | Aegilops tauschii | -1.133 |
| 47  | gi|15237579  | RNA-binding protein NOB1 | 67.08/5.55 | 61    | 14 | Arabidopsis thaliana | 2.558 |
| 62  | gi|355477483 | F-box family protein | 19.67/4.56 | 63    | 2 | Medicago truncatula | -1.133 |
| 72  | gi|514725733 | Predicted: chloroplast stem-loop binding protein of 41 kDa, chloroplastic-like | 41.49/6.41 | 203   | 6 | Setaria italica | -1.133 |

**Others proteins**

| No. | Accession | Description | Fold Change | p-Value | Score | Organism | Score |
|-----|-----------|-------------|-------------|---------|-------|----------|-------|
| 20  | gi|508727025 | Phosphate transporter traffic facilitator isoform 2 | 34.31/6.46 | 62    | 6 | Theobroma cacao | 4.787 |
| 60  | gi|502120213 | Predicted: flocculation protein FLO11-like isoform X2 | 66.28/10.59 | 69    | 4 | Cicer arietinum | 5.023 |
Table 2. Identification of DEPs of *A. ilicifolius* roots with an expression change greater than 2.0-fold change under tidal flooding stress

| Spot  | Accession (gb) | Protein Name c | Thero. d kDa/pI | Exper. e kDa/pI | Score | MP | Species h | SF vs. CK |
|-------|----------------|----------------|-----------------|-----------------|-------|----|-----------|----------|
| TCA cycle | | | | | | | | |
| R37   | gi|226503019 | Malate dehydrogenase, cytoplasmic Carbon and energy metabolism | 35.84/5.76 | 13.42/5.98 | 127 | 2 | Zea mays | 1.359 |
| R10   | gi|460407876 | V-type proton ATPase subunit d2-like | 41.3/4.9 | 36.53/4.62 | 154 | 6 | Solanum lycopersicum | -3.684 |
| R11   | gi|449454235 | Predicted: V-type proton ATPase subunit B 1-like | 54.36/4.96 | 52.22/4.67 | 393 | 9 | Cucumis sativus | 6.056 |
| R12   | gi|470108902 | Predicted: V-type proton ATPase subunit B2-like | 54.61/5.07 | 53.04/4.70 | 72 | 8 | Fragaria vesca subsp. vesca | 4.146 |
| R16   | gi|490262869 | ATP synthase subunit D, partial | 19.24/5.21 | 14.44/5.18 | 102 | 2 | Medicago truncatula | 3.827 |
| R19   | gi|355479515 | Adenosine kinase | 38.08/5.08 | 33.59/5.14 | 104 | 4 | Hydnora visseri | 1.438 |
| R22   | gi|473798701 | ATP synthase subunit beta, mitochondrial | 57.83/5.25 | 49.52/5.15 | 719 | 10 | Triticum urartu | 1.402 |
| R26   | gi|346683384 | ATPase subunit 1 | 55.24/5.58 | 61.31/6.16 | 435 | 11 | Cucumis sativus | 2.206 |
| R29   | gi|449434570 | Predicted: ATP synthase subunit beta, mitochondrial-like | 59.89/0.00 | 49.56/5.27 | 121 | 16 | Cucumis sativus | 5.272 |
| R30   | gi|110288667 | Enolase, putative, expressed | 51.89/5.72 | 52.95/5.29 | 492 | 11 | Oryza sativa Japonica Group | 1.581 |
| R32   | gi|398363571 | Fructokinase | 34.69/5.49 | 28.6/5.46 | 123 | 4 | Actinidia delicosa | 3.000 |
| R38   | gi|527196189 | Nucleoside diphosphate kinase | 16.48/6.43 | 52.61/6.17 | 143 | 4 | Genlisea aurea | 2.445 |
| Amino acid and protein metabolism | | | | | | | | |
| R7    | gi|223532621 | Proteasome subunit beta type 6,9, putative | 24.91/5.17 | 18.50/4.78 | 109 | 3 | Ricinus communis | -4.315 |
| R13   | gi|315307966 | Heat shock protein 90-1 | 80.45/4.96 | 67.92/4.85 | 155 | 5 | Nicotiana attenuata | 5.239 |
| R14   | gi|527187624 | Heat shock protein 70 | 71.62/5.06 | 67.43/4.92 | 513 | 22 | Genlisea aurea | 8.424 |
|   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|
| R15 | gi|223535705 | 60S ribosomal protein L23, putative | 7.17/11.09 | 11.34/5.12 | 74 | 4 | Ricinus communis | 4.451 |
| R23 | gi|508784980 | TCP-1/cpn60 chaperonin family protein | 64.51/5.62 | 56.16/4.98 | 159 | 6 | Theobroma cacao | 2.581 |
| R27 | gi|527189531 | Protein disulfide-isomerase, partial | 38.13/5.26 | 31.65/5.33 | 109 | 4 | Genlisea aurea | 4.561 |
| R33 | gi|332656685 | S-adenosylmethionine synthase 2 | 43.63/5.67 | 44.43/5.56 | 346 | 7 | Arabidopsis thaliana | 2.857 |
| R34 | gi|351722651 | Glutamine synthetase cytosolic isozyme 1 | 38.99/5.46 | 32.95/5.74 | 154 | 5 | Glycine max | 7.338 |
| R40 | gi|351722651 | Glutamine synthetase cytosolic isozyme 1 | 38.99/5.46 | 25.31/6.17 | 186 | 6 | Glycine max | 3.97 |
|   |   | Stress and defense |   |   |   |   |   |   |
| R1 | gi|223529085 | Peroxidase, putative | 19.45/8.6 | 32.03/4.05 | 74 | 1 | Ricinus communis | -1.056 |
| R17 | gi|474311703 | L-ascorbate peroxidase 1, cytosolic | 27.56/5.85 | 23.23/5.14 | 160 | 4 | Triticum urartu | 1.219 |
| R20 | gi|527187175 | Monodehydroascorbate reductase | 47.2/5.82 | 39.32/5.12 | 118 | 3 | Genlisea aurea | 4.013 |
| R31 | gi|427199300 | Thioredoxin | 13.67/5.76 | 11.16/5.73 | 183 | 4 | Ipomoea batatas | 1.824 |
| R36 | gi|223551378 | Catalase, putative | 113.36/6.84 | 38.22/5.88 | 234 | 11 | Glycine max | 3.83 |
|   |   | Transcription and signal transduction |   |   |   |   |   |   |
| R2 | gi|526117762 | 14-3-3 protein | 29.47/4.79 | 23.65/4.48 | 132 | 2 | Vitis vinifera | 1.661 |
| R3 | gi|350539221 | 14-3-3 protein 7 | 28.91/4.96 | 24.95/4.33 | 110 | 2 | Solanum lycopersicum | 1.531 |
| R4 | gi|543176851 | 14-3-3 protein | 29.26/4.66 | 24.57/4.41 | 277 | 7 | Phaseolus vulgaris | -1.475 |
| R21 | gi|527203530 | Actin-97 | 41.95/5.37 | 41.35/5.15 | 698 | 14 | Genlisea aurea | 2.133 |
| R24 | gi|473749533 | NuA3 HAT complex component NTO1 | 103.46/7.88 | 12.95/3.36 | 63 | 17 | Triticum urartu | 4.488 |
| R25 | gi|375968572 | SKP1 protein | 17.63/0.00 | 14.72/5.37 | 141 | 5 | Nicotiana tabacum | -1.982 |
| R28 | gi|527203530 | Actin-97 | 41.95/5.37 | 41.29/5.26 | 706 | 16 | Genlisea aurea | 3.076 |
| R8 | gi|508724744 | Pathogen-related protein | 27.54/5.10 | 25.65/4.76 | 64 | 4 | Theobroma | -1.063 |
| ID | Accession number | Description | MFR (A/B) | MFO (A/B) | Score | Organism |
|----|------------------|-------------|-----------|-----------|-------|-----------|
| R39 | gi|15239652 | Flavodoxin-like quinone reductase 1 | 21.40/5.96 | 15.6/6.43 | 84 | 2 | cacao |
| Unknown proteins |  |  |  |  |  |  |  |  |
| R5 | gi|125562472 | Hypothetical protein OsI_30174 | 16.21/8.51 | 59.29/4.46 | 73 | 6 | Arabidopsis thaliana |
| R6 | gi|226521422 | Predicted protein | 88.7/5.56 | 59.7/4.49 | 68 | 14 | Oryza sativa Indica Group |
| R9 | gi|1527206839 | Hypothetical protein M569_02468 | 27.34/9.01 | 32.97/4.88 | 76 | 4 | Micromonas sp. RCC299 |
| R18 | gi|557113120 | Hypothetical protein EUTSA_v10025711mg | 34.88/6.07 | 28.49/5.14 | 139 | 4 | Genlisea aurea |
| R35 | gi|557531169 | Hypothetical protein CICLE_v10012166mg | 36.16/9.43 | 25.17/5.9 | 140 | 3 | Eutrema salsugineum |
|  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |
Table 3. Identification of DEPs of *A. mollis* leaves with an expression change greater than 2.0-fold change under tidal flooding stress

| Spot | Accession (gb) | Protein Name | Thero. d kDa/pI | Exper. e kDa/pI | Score t | MP | Species | SF vs. CK |
|------|----------------|--------------|------------------|------------------|---------|----|---------|-----------|
| 20   | gi|543176923      | Oxygen-evolving enhancer protein 1 | 35.20/6.08       | 19.74/5.42      | 170    | 4   | *Phaseolus vulgaris* | -2.242    |
| 26   | gi|474445723      | Phosphoglycolate phosphatase | 33.22/8.87       | 24.64/5.41      | 64     | 4   | *Triticum urartu*    | -1.575    |
| 33   | gi|223539254      | Oxygen-evolving enhancer protein 2, chloroplast precursor, putative | 28.76/8.63       | 13.68/5.79      | 130    | 4   | *Ricinus communis*   | -2.109    |
| 43   | gi|474121685      | Chlorophyll a-b binding protein 8, chloroplastic | 29.29/8.69       | 11.86/5.78      | 109    | 3   | *Triticum urartu*    | -2.066    |
| 52   | gi|223551247      | Ferredoxin-NADP reductase, putative | 40.74/8.70       | 14.35/6.50      | 69     | 9   | *Ricinus communis*   | 4.265     |
| 63   | gi|527197786      | Cytochrome b6-f complex iron-sulfur subunit 1, chloroplastic, partial | 24.25/8.48       | 11.51/6.78      | 1.9    | 4   | *Genlisea aurea*      | 2.970     |
| 64   | gi|1330318806     | Photosystem I reaction center subunit iv b | 11.37/9.88       | 12.76/6.95      | 67     | 4   | *Camellia sinensis*   | 2.491     |
| 21   | gi|1399139356     | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial | 51.49/6.46       | 34.61/4.81      | 654    | 19  | *Anisochilus pallidus* | -4.053    |
| 21   | gi|1335059563     | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | 34.09/7.83       | 34.19/4.74      | 245    | 3   | *Humbertia madagascariensis* | -3.850    |
| 21   | gi|502137718      | Predicted: ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic-like | 48.07/8.47       | 40.24/5.02      | 298    | 7   | *Cicer arietinum*     | 7.049     |
| 21   | gi|4888888859     | Chloroplast rubisco activase 1 | 48.37/7.66       | 39.76/5.09      | 300    | 4   | *Sagittaria graminea* | 2.472     |
| 21   | gi|4888888859     | Chloroplast rubisco activase 1 | 48.37/7.66       | 40.27/5.26      | 204    | 5   | *Sagittaria graminea* | 1.432     |
|   | gi  | Description                                                                 | Expression Ratio | Protein ID | Species                          | Cutoff | p-value |
|---|-----|------------------------------------------------------------------------------|------------------|------------|-----------------------------------|--------|---------|
| 22 | 355488628 | Ribulose bisphosphate carboxylase large chain                               | 19.46/4.87       | 48.33/5.43 | Medicago truncatula               | 72     | -4.455  |
| 25 | 452119476 | Ribulose bisphosphate carboxylase large subunit, partial (chloroplast) Phosphoribulokinase | 47.76/0.00       | 13.67/5.68 | Ulva reticulata x Ulva taeniata   | 81     | -3.093  |
| 27 | 355513999 | Chloroplast rubisco activase 2                                              | 46.00/6.68       | 35.60/5.34 | Medicago truncatula               | 118    | -1.498  |
| 28 | 488888860 | Chloroplast rubisco activase 2                                              | 36.40/6.33       | 34.77/5.37 | Sagittaria graminea               | 178    | 2.933   |
| 35 | 488888859 | Chloroplast rubisco activase 1                                              | 48.37/7.66       | 35.45/5.50 | Sagittaria graminea               | 283    | 2.256   |
| 36 | 488888860 | Chloroplast rubisco activase 2                                              | 36.40/6.33       | 35.35/5.59 | Sagittaria graminea               | 212    | 1.143   |
| 40 | 340511990 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial(chloroplast) | 50.77/6.23       | 45.85/5.62 | Scutellaria minor                 | 337    | -1.418  |
| 51 | 413953335 | Transketolase isoform 2                                                     | 69.06/5.46       | 68.55/6.07 | Zea mays                         | 167    | -1.502  |
| 66 | 399139488 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial(chloroplast) | 51.66/6.19       | 45.56/6.70 | Crossandra infundibuliformis      | 435    | 1.249   |

**Carbon metabolism**

|   | gi  | Description                                                                 | Expression Ratio | Protein ID | Species                          | Cutoff | p-value |
|---|-----|------------------------------------------------------------------------------|------------------|------------|-----------------------------------|--------|---------|
| 13 | 404551307 | Glyceraldehyde-3-phosphate dehydrogenase, partial                           | 13.46/6.89       | 49.62/6.82 | Agave x ajoensis                  | 133    | -2.462  |
| 47 | 317373797 | Chloroplast phosphoglycerate kinase 3                                       | 50.28/6.69       | 46.51/5.79 | Helianthus annuus                 | 284    | -1.571  |
| 57 | 449450436 | Predicted: glutamate-glyoxylate aminotransferase 2-like                     | 52.90/5.62       | 56.93/6.31 | Cucumis sativus                   | 260    | -3.996  |
| 61 | 530684266 | Fructose-bisphosphate aldolase                                              | 42.21/6.38       | 29.80/6.47 | Oryza sativa Japonica Group       | 345    | 1.987   |
| 65 | 332645863 | Triosephosphate isomerase                                                   | 27.38/5.39       | 19.18/4.00 | Arabidopsis thaliana             | 151    | 4.485   |

**TCA cycle**

|   | gi  | Description                                                                 | Expression Ratio | Protein ID | Species                          | Cutoff | p-value |
|---|-----|------------------------------------------------------------------------------|------------------|------------|-----------------------------------|--------|---------|
| 45 | 461488119 | Succinyl-CoA ligase beta-chain                                               | 45.40/5.98       | 36.07/6.05 | Oryza sativa Japonica Group       | 171    | 3.395   |
|   | gi   | Function                                      | Identity 1   | Identity 2   | Identity 3   | Identity 4   | Identity 5   |
|---|------|-----------------------------------------------|--------------|--------------|--------------|--------------|--------------|
| 54 | gi433335660 | Malate dehydrogenase                          | Malate dehydrogenase | 36.04/6.11   | 294          | Brassica oleracea | 1.040       |
| 62 | gi475577109 | Malate dehydrogenase 1, mitochondrial         | Malate dehydrogenase 1, mitochondrial | 34.93/5.26 | 96 | Aegilops tauschii | 2.970       |
| 16 | gi408899417 | AtpA, partial (chloroplast)                   | AtpA, partial (chloroplast) | 55.13/5.53 | 315 | Mammea americana | 1.179       |
| 23 | gi408899391 | AtpA, partial (chloroplast)                   | AtpA, partial (chloroplast) | 55.08/5.08 | 514 | Erythroxylum aroelatum | 1.179       |
| 29 | gi546138044 | ATP synthase CF1 alpha subunit (chloroplast)  | ATP synthase CF1 alpha subunit (chloroplast) | 56.14/5.70 | 163 | Cocos nucifera | 1.179       |
| 30 | gi402243685 | ATP synthase beta subunit, partial (chloroplast) | ATP synthase beta subunit, partial (chloroplast) | 49.69/5.11 | 737 | Flindersia laevicarpa | 1.179       |
| 31 | gi410176162 | ATP synthase CF1 beta subunit (chloroplast)   | ATP synthase CF1 beta subunit (chloroplast) | 53.54/5.09 | 939 | Origanum vulgare subsp. vulgare | 1.179       |
| 37 | gi223535342 | Alcohol dehydrogenase, putative               | Alcohol dehydrogenase, putative | 41.61/8.61 | 103 | Ricinus communis | 1.179       |
| 53 | gi330254337 | NAD(P)-binding Rossmann-fold-containing protein | NAD(P)-binding Rossmann-fold-containing protein | 34.97/8.37 | 102 | Arabidopsis thaliana | 1.179       |

Energy metabolism

|   | gi   | Function                                      | Identity 1   | Identity 2   | Identity 3   | Identity 4   | Identity 5   |
|---|------|-----------------------------------------------|--------------|--------------|--------------|--------------|--------------|
| 2  | gi226500014 | 3-beta hydroxysteroid dehydrogenase/isomerase family protein | 32.73/8.34 | 77 | Zea mays | -1.236  |
| 10 | gi222863465 | Glutamine synthetase family protein           | 48.20/6.48 | 211 | Populus trichocarpa | 1.857  |
| 11 | gi223551115 | Proteasome subunit alpha type, putative       | 30.64/4.89 | 192 | Ricinus communis | -2.136  |
| 17 | gi226499880 | Stromal 70 kDa heat shock-related protein     | 74.85/5.08 | 259 | Zea mays | -2.046  |
| 32 | gi392465167 | Heat shock protein 70                         | 71.46/5.14 | 140 | Nicotiana tabacum | 2.392  |
| 34 | gi527197598 | Cysteine synthase                             | 34.65/5.41 | 100 | Genlisea aurea | -3.840  |
| 38 | gi226508704 | Elongation factor Tu                          | 50.79/6.07 | 184 | Zea mays | -1.312  |
| 39 | gi449440632 | Predicted: elongation factor Tu,              | 51.89/5.90 | 94 | Cucumis sativus | -1.458  |
| 41 | gi|508784980 | TCP-1/cpn60 chaperonin family protein | 64.51/5.62 | 61.78/5.48 | 233 | 3 | Theobroma cacao | -1.794 |
| 44 | gi|449506050 | Predicted: glutamine synthetase nodule isozyme-like | 39.30/5.59 | 36.26/6.00 | 75 | 4 | Cucumis sativus | 3.714 |
| 48 | gi|543176708 | ATP sulfurylase 2-like protein | 40.12/0.00 | 45.36/5.86 | 253 | 11 | Phaseolus vulgaris | -1.428 |
| 50 | gi|460415276 | Predicted: adenosyl homocysteine-like Arginase 2 | 53.69/5.78 | 51.09/6.03 | 355 | 9 | Solanum lycopersicum | -1.435 |
| 56 | gi|350538867 | Stress and defense Predicted: peroxiredoxin-2E, chloroplastic-like | 23.44/7.67 | 29.05/7.0 | 125 | 1 | Cicer arietinum | -1.189 |
| 1 | gi|502111694 | Cicer arietinum Predicted: peroxiredoxin-2E, chloroplastic-like | 23.44/7.67 | 36.6/4.86 | 125 | 1 | Cicer arietinum | -5.574 |
| 7 | gi|511774224 | 2-Cys peroxiredoxin, partial | 25.60/8.51 | 20.94/4.93 | 398 | 11 | Nicotiana benthamiana | -2.897 |
| 12 | gi|223533515 | Peroxidase 12 precursor, putative | 39.40/7.55 | 35.09/4.94 | 110 | 2 | Ricinus communis | 1.136 |
| 24 | gi|145323784 | Arabidopsis thaliana L-ascorbate peroxidase 1 | 27.79/5.41 | 13.12/5.50 | 376 | 6 | Arabidopsis thaliana | -1.521 |
| 42 | gi|460384911 | 2.169 | | | | | | Setaria italica | 2.957 |
| 46 | gi|222856181 | 3.093 | | | | | | Setaria italica | -1.598 |
| 49 | gi|508777590 | Monodehydroascorbate reductase 6 isoform 4 | 53.16/8.80 | 46.57/6.16 | 337 | 5 | Theobroma cacao | -1.598 |
| 60 | gi|1330555786 | Glutathione S-transferase phi 8 | 29.27/0.00 | 18.84/6.43 | 83 | 3 | Arabidopsis thaliana | 2.957 |
| 8 | gi|514824684 | Transcription and signal transduction Predicted: fanconi anemia group I protein homolog | 153.86/8.0 | 22.31/5.07 | 61 | 13 | Setaria italica | -1.455 |
Table 4. Identification of DEPs of *A. mollis* roots with an expression change greater than 2.0-fold change under tidal flooding stress

| Spot | Accession (gb) | Protein Name | Thero. kDa/pI | Exper. kDa/pI | Score | MP | Species | SF vs. CK |
|------|----------------|--------------|---------------|---------------|-------|----|---------|----------|
|      | gi|113622845      | Os08g0120000  | 31.84/8.81    | 46.91/5.23   | 62  | 5   | *Oryza sativa* Japonica Group | 1.372    |
|      | gi|470107271      | Predicted: succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial-like | 45.39/5.87 | 34.20/5.76 | 93  | 4   | *Fragaria vesca* subsp. *vesca* | -3.345   |
|      | gi|475610756      | Succinate dehydrogenase (ubiquinone) flavoprotein subunit, mitochondrial | 81.40/6.24 | 57.35/6.09 | 98  | 5   | *Aegilops tauschii*          | -2.810   |
|      | gi|123547542      | NADH dehydrogenase, putative | 19.14/4.76 | 12.75/4.50 | 77  | 1   | *Ricinus communis*          | -2.186   |
| R2 | gi|223539983 | Alpha-galactosidase/alpha-n-acetylgalactosaminidase, putative | 40.06/5.19 | 40.56/4.28 | 125 | 6 | *Ricinus communis* | -1.844 |
| R10 | gi|514802088 | Predicted: beta-glucosidase 12-like | 51.94/6.73 | 53.37/4.52 | 61 | 12 | *Setaria italica* | -3.641 |
| R20 | gi|355479515 | Adenosine kinase | 38.08/5.08 | 25.56/5.20 | 103 | 6 | *Medicago truncatula* | 1.701 |
| R26 | gi|470124373 | Predicted: beta-galactosidase 13-like | 87.36/8.91 | 60.62/5.27 | 63 | 9 | *Fragaria vesca* subsp. *vesca* | -6.185 |
| R30 | gi|350538295 | Enolase | 48.05/5.68 | 49.90/5.73 | 301 | 8 | *Solanum lycopersicum* | 5.906 |
| R35 | gi|550346968 | UDP-glucose 4-epimerase family protein | 38.44/5.66 | 31.38/5.99 | 108 | 7 | *Populus trichocarpa* | -2.385 |
| R36 | gi|223525768 | Alcohol dehydrogenase, putative | 41.88/5.98 | 43.45/6.38 | 106 | 4 | *Ricinus communis* | 1.372 |
| R39 | gi|390098824 | Triosephosphate isomerase cytosolic isoform-like | 27.31/5.72 | 16.51/4.00 | 207 | 6 | *Capsicum annuum* | -2.761 |
| R40 | gi|111467928 | Cytochrome b | 45.52/9.85 | 24.80/6.69 | 61 | 3 | *Acutodesmus obliquus* | -2.804 |
| R42 | gi|460405093 | Predicted: probable aldo-keto reductase 4-like | 60.97/5.51 | 31.89/6.22 | 76 | 5 | *Solanum lycopersicum* | -1.893 |
| R43 | gi|1508708397 | Thiamin diphosphate-binding fold (THDP-binding) superfamily protein isoform 2 | 36.04/8.67 | 35.88/6.31 | 63 | 5 | *Theobroma cacao* | -2.545 |
| R44 | gi|223525768 | Alcohol dehydrogenase, putative | 41.88/5.98 | 43.26/6.32 | 61 | 5 | *Ricinus communis* | 2.574 |
| R45 | gi|407369264 | Alcohol dehydrogenase, partial | 20.74/6.08 | 50.46/6.32 | 76 | 1 | *Pinus taiwaniensis* | 1.412 |

Amino acid and protein metabolism

| R1 | gi|460411270 | Predicted: 60S acidic ribosomal protein P2B-like isoformR1 | 11.41/4.55 | 9.28/4.05 | 86 | 1 | *Solanum lycopersicum* | -1.880 |
| R11 | gi|432140649 | Heat shock protein 70 | 74.29/5.26 | 72.91/4.58 | 475 | 16 | *Lactuca sativa* | 3.076 |
| R13 | gi|430763366 | Polyubiquitin 14, partial | 15.57/5.74 | 28.74/4.83 | 68 | 2 | *Cornus kousa* | -3.358 |
| R14 | gi|502126081 | Predicted: proteasome subunit alpha type-1-B-like | 31.59/4.94 | 30.00/4.90 | 100 | 5 | *Cicer arietinum* | -1.714 |
| R15 | gi|514761174 | Predicted: 26S protease regulatory subunit 6A homolog | 47.99/4.94 | 43.41/4.77 | 308 | 14 | *Setaria italica* | 1.053 |
| R23 | gi|334184654 | Heat shock protein 60-2 | 61.78/6.08 | 60.46/5.14 | 202 | 11 | Arabidopsis thaliana | -3.003 |
| R24 | gi|413956514 | Glutamine synthetase3 | 18.23/6.92 | 35.05/5.36 | 79 | 6 | Zea mays | -2.010 |
| R25 | gi|330252829 | 20S proteasome alpha subunit G1 | 27.64/5.93 | 38.92/5.39 | 62 | 10 | Arabidopsis thaliana | 1.143 |
| R27 | gi|508778822 | Tetratricopeptide repeat (TPR)-like superfamily protein, putative | 64.56/8.70 | 50.52/5.61 | 61 | 14 | Theobroma cacao | 1.036 |
| R29 | gi|355429958 | Putative S-adenosyl-L-homocysteinase | 61.87/5.69 | 45.20/5.89 | 75 | 8 | Linum usitatissimum | 2.505 |
| R31 | gi|527194033 | S-formylglutathione hydrolase, partial | 24.93/6.22 | 27.19/6.21 | 69 | 2 | Genlisea aurea | -2.060 |
| R38 | gi|502137510 | Predicted: 60S ribosomal export protein NMD3-like | 59.49/6.07 | 15.16/6.32 | 71 | 14 | Cicer arietinum | -2.219 |

**Stress and defense**

| R2 | gi|511774224 | 2-Cys peroxiredoxin, partial | 25.60/8.51 | 15.21/4.49 | 77 | 5 | Nicotiana benthamiana | -2.780 |
| R17 | gi|440573478 | Tau class glutathione S-transferase | 27.80/7.62 | 48.05/4.92 | 63 | 8 | Pinus tabuliformis | -3.345 |
| R4 | gi|297333574 | GF14 omega | 29.37/4.70 | 21.33/4.35 | 112 | 4 | Arabidopsis lyrata subsp. lyrata | -2.804 |
| R8 | gi|460377572 | Predicted: 14-3-3-like protein-like | 29.22/4.69 | 21.08/4.54 | 84 | 6 | Solanum lycopersicum | -1.246 |
| R9 | gi|226498758 | Inositol-tetrakisphosphate 1-kinase 3 | 37.72/8.59 | 41.17/4.47 | 63 | 8 | Zea mays | -2.060 |
| R12 | gi|413942896 | Profilin-4 | 14.21/4.63 | 10.66/4.71 | 71 | 2 | Zea mays | 4.448 |
| R21 | gi|508715249 | Ran-binding protein 1 b isoform 1 | 25.21/4.70 | 29.56/4.99 | 79 | 2 | Theobroma cacao | -1.913 |
| R31 | gi|508786508 | Cell division control 6 isoform 7 | 47.05/8.95 | 21.75/6.48 | 66 | 14 | Theobroma cacao | -1.658 |
| R32 | gi|223549247 | ATP-dependent RNA helicase, putative | 78.24/8.86 | 23.64/6.31 | 62 | 16 | Ricinus communis | 1.741 |
| R34 | gi|186510546 | Ankyrin repeat family protein | 87.56/6.52 | 26.05/6.27 | 67 | 11 | Arabidopsis thaliana | 2.089 |

**Photosynthesis**

| R18 | gi|543177187 | RuBisCO large subunit-binding protein subunit alpha, belongs to the | 61.28/0.00 | 54/4.8 | 147 | 4 | Phaseolus vulgaris | -1.076 |
| Spot | Accession | Description                  | Theoretical Mass (kDa) | Experimental Mass (kDa) | pI | Species                           | Log2 (fold change) |
|------|-----------|------------------------------|------------------------|------------------------|----|-----------------------------------|--------------------|
| R6   | gi|514802368 | Predicted: endochitinase A-like | 30.18/8.55             | 39.52/7.00             | 83  | Setaria italica                   | 3.202              |
| R16  | gi|515242097 | Putative villin              | 108.44/5.2             | 49.09/4.86             | 60  | Arabidopsis thaliana              | -2.169             |
|      |           |                              |                        |                        |     |                                   |                    |
| R19  | gi|508787331 | Ferritin 4                   | 30.44/6.56             | 17.58/4.99             | 116 | Theobroma cacao                   | 5.694              |
| R5   | gi|300256770 | Hypothetical protein         | 87.21/5.31             | 32.42/4.25             | 61  | Volvox carteri f. nagariensis     | -3.003             |
| R41  | gi|162688983 | Predicted protein            | 38.61/8.27             | 30.98/6.42             | 63  | Physcomitrella patens subsp.      | -1.472             |

*a* The spot number corresponding to the number listed in the table 1-4. R represents the root tissue. Underlined numbers represent *A. mollis* tissues.

*b* Database accession numbers (gb) according to NCBI nr.

*c* The name of proteins was identified by LC-MALDI-TOF/TOF.

*d* Theoretical mass (kDa) and pI of identified proteins. Theoretical values were retrieved from the NCBI nr database.

*e* Experimental mass (kDa) and pI of identified proteins. Experimental values were calculated by using PDquest software and standard molecular mass markers.

*f* The Mascot searched score against the database NCBI nr.

*g* Number of matched peptide fragments.

*h* The species that has the high homology of the identified protein.

*i* Log2 (fold change) values between the different treatments. SF vs CK means soil flooding treatment vs control group.
2.5. Identification of hub proteins in Acanthus species

Because of the lack of genome information on *A. ilicifolius* and *A. mollis*, the DEPs of the two species were annotated based on the existing NR database. Based on our previous studies (Li et al. 2020) and homologous protein distribution analysis (Supplementary Fig. S3), *Arabidopsis thaliana* was used to assemble the PPI network of *A. ilicifolius* and *A. mollis*. The top-ten hub proteins were identified with a degree score of CytoHubba and displayed in Figure 6. The hub proteins of *A. ilicifolius* tissues were mostly associated with carbon and energy metabolism (Fig. 6A, B), whereas those of the *A. mollis* tissues were mostly associated with photosynthesis and photorespiration and the TCA cycle (Fig. 6C, D).

![Fig. 6. Top 10 hub proteins in network of (A) *A. ilicifolius* leaves, (B) *A. ilicifolius* roots, (C) *A. mollis* leaves, (D) *A. mollis* roots ranked by Matthews correlation coefficient (MCC) method. R represents the root tissue. Underlined numbers represent *A. mollis* tissues.](image)

2.6. Tidal flooding stress influences the energy status level of *A. ilicifolius* and *A. mollis*

The further comparison demonstrated that *A. mollis* had a higher AMP, ADP, and ATP content than *A. ilicifolius* in the control group (Fig. 7A-C). *A. ilicifolius* promptly responded to flooding stress by significantly increasing ADP and ATP contents in the leaves (Fig. 7B-C). However, AMP and ATP contents were significantly decreased in *A. mollis* roots (Fig. 7B-C) under tidal flooding stress. The energy charge represents the energy status of biological cells [24]. Whereas the energy charge of *A. ilicifolius* roots was significantly increased under tidal flooding stress, it was significantly decreased in *A. mollis* tissues (Fig. 7D).
Fig. 7. Effects of tidal flooding stress on (A) AMP content, (B) ADP content, (C) ATP content, and (D) energy charge of *A. ilicifolius* and *A. mollis*. * and ** indicate significant difference at the 0.05 level and the 0.01 level, respectively. CK and SF represent the control group and soil tidal flooding stress, respectively.

2.7. Tidal flooding stress influences the total soluble sugar and starch contents of Acanthus species

There was no significant change in the content of total soluble sugar and starch of *A. ilicifolius* tissues under tidal flooding stress (Fig. 8A-B). Nevertheless, there was significant tidal flooding tolerance in the ratio of soluble sugar to starch in *A. ilicifolius* tissues (Fig. 8C). The total soluble sugar
content, starch content, and the ratio of soluble sugar to starch were lower in *A. mollis* tissues than in the control group, except for the ratio of soluble sugar to starch in the leaves (Fig. 8A-C).

Fig. 8. The concentration of soluble sugar, starch and the ratio of soluble sugar to starch for *A. ilicifolius* and *A. mollis* under tenth day tidal flooding stress. * and ** indicate significant difference at the 0.05 level and the 0.01 level, respectively. CK and SF represent control and soil tidal flooding stress, respectively.

2.8. qRT-PCR validation of the abundance of proteins

The genes were selected for real-time PCR analysis to confirm the reliability of protein abundance in this study. The details of these genes and their specific primers are shown in Supplemental Table S1. The results showed that most of the gene expressions in *A. ilicifolius* and *A. mollis* were strongly correlated with protein abundance (Fig. 9), confirming the reliability of the protein data.
Fig. 9. Proteins levels confirmation by mRNA. Ten genes (Table S1) of (A) *A. ilicifolius* and (B) *A. mollis* were selected for analysis the correlation between the protein abundance and mRNA levels. Protein abundance is depicted by square and mRNA levels are depicted by circle. Red indicates up-regulation and blue indicates down-regulation.

3. Discussion

3.1 Differences in tissue tolerance between Acanthus species

Unlike previous findings in the mangrove, *A. marina*, seedlings [25], the upper and lower epidermises of *A. ilicifolius* showed no change with prolonged waterlogging duration in the present study (Fig. 1B2, B3). The leaf anatomical features of *A. mollis* were also relatively less susceptible to tidal flooding stress within a short span. The leaf anatomy plays an important role in determining photosynthetic capacity. Herbaceous plants with high photosynthetic capacity usually have thinner epidermis, leading to high values of mesophyll conductance [26]. The mangrove leaf exhibited a range of xeromorphic features, including thick epidermis and wax coatings [25]. Therefore, like other mangrove plants, *A. ilicifolius* leaves are likely to regulate the Calvin cycle to resist the tidal flooding stress (Table 1). A comparative analysis showed that palisade and spongy tissue that were loosely arranged with large spaces and epidermis were thinner in *A. mollis* leaf, making CO₂ entry easier.

*A. ilicifolius*, mainly distributed in the foreshore seaward region, was found to develop a high percentage of schizogenous aerenchyma to facilitate efficient internal oxygen transfer [27]. According to previous study, the mangrove species appeared to higher waterlogging tolerance when the aerenchyma formation was induced [28]. The aortic root anatomy of *A. ilicifolius* was not affected by the tidal flooding stress. The special anatomical structure of roots was not the main reason for *A. ilicifolius* to tolerance tidal flooding stress at the early stage. Water and minerals transport from the root system to the aerial portions via the xylem tissue. The phloem translocates photosynthetic products from mature leaves to roots and redistributes water and various compounds throughout the plant body [29]. In *A. mollis*, the aortic root anatomy exhibited broken xylem, phloem, and periderm tissues, indicating a negative influence on the allocation and partitioning of photosynthetic products (Fig. 1 C4, D4).

3.2 Effect of tidal flooding on the photosynthesis of Acanthus species

The proportion of photosynthesis-related proteins within the total DEPs of *A. ilicifolius* leaves was close to that of *A. mollis* leaves (Fig. 5). Most hub proteins of *A. mollis* leaves were associated with photosynthesis (Fig. 6C). The abundance of oxygen-evolving enhancer protein (OEE, spot 18) showed an increasing trend in *A. ilicifolius* leaves under tidal flooding stress. Oxygen-evolving complex (OEC) proteins are degraded and release OEE as a degradation product to promote the plant to adapt to the adverse conditions [30]. OEE is a subunit of the OEC of photosystem II in the chloroplast [31] considered to be directly involved in photosynthesis. It is suggested that decreased OEE abundance (spot 20, 33) might negatively affect *A. mollis* leaves.

Chlorophyll a/b-binding protein (light-harvesting complex, LHC) captures light in the light reaction [32]. Root hypoxia suppressed photosynthetic activity by decreasing the expression of LHC in pea leaves [33]. Most of chlorophyll a/b-binding proteins increased in *A. ilicifolius* leaves (spot 12,
13, 58) but decreased in A. mollis leaves (spot 43) under tidal flooding stress. However, electron transport chain proteins, such as ferredoxin-nicotinamide adenine dinucleotide phosphate (NADP) reductase (spot 52), cytochrome b6-f complex iron-sulphur subunit 1 (spot 63), and photosystem I reaction center subunit IV b (spot 64), increased in A. mollis leaves, promoting photosynthetic electron transport under tidal flooding stress [31]. One fraction of the captured light energy is used to reduce NADP to reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the other fraction is used for light-dependent ATP synthesis. The proteomic data showed that chloroplast ATP synthesis was decreased in A. mollis leaves (spot 23, 29, 30, 31). ATP-dependent zinc metalloprotease FTSH2 (FTSH2, spot 41, 42), which is involved in the turnover of the ΦPSII reaction center D1 protein [35], was increased in maize to protect chloroplast photosynthesis under heat stress [35]. Herein, increased FtsH2 abundance had a positive effect on tidal flooding tolerance of A. ilicifolius leaves.

3.3 Effect of tidal flooding on carbon and energy metabolism of Acanthus species

It is well known that the photosynthetic system and its maintenance can severely affect plant survival under an abiotic stress environment [36]. Therefore, to meet the increased demands for survival, plants change their energy metabolism pathways.

3.3.1. Calvin cycle

The reactions catalyzing the reduction of CO$_2$ to carbohydrates are coupled to the consumption of ATP and NADPH by enzymes found in the stroma, the soluble phase of chloroplasts. Therefore, we compared Calvin cycle-related proteins between the two species to determine their CO$_2$ fixation ability under tidal flooding stress. The activation state of Rubisco, a key enzyme in the Calvin cycle, is regulated by Rubisco activase [37]. RuBisCO activase increased in both A. ilicifolius (spot 14, 15, 21, 28, 35 and 36) and A. mollis (spot 14, 15, 21, 28, 35, and 36). The abundance of RuBisCO large subunits increased in A. ilicifolius (spots 7, 9, and 15) but decreased in A. mollis (spots 3, 5, 22, 25, and 40); the protein abundance showed a different change between the two species. A similar result was observed in Trifolium species, the waterlogging sensitive species exhibited a higher reduction of Rubisco large subunits expression [36].

3.3.2. Sugar metabolism

CO$_2$ fixation is performed through the Calvin cycle to drive sugar production and energy storage in plants [35]. A higher soluble sugar concentration (Figure 8A) in flooded A. ilicifolius plant is not solely due to photosynthesis but also the conversion of carbohydrates from starch to sugar (Figure 8C). We found that A. ilicifolius roots in the abundance of fructokinase (spot R32) exhibited a 3.0-fold increase compared with controls in response to tidal flooding treatment. Fructokinase regulates starch synthesis coordinately with sucrose synthase and plays a key role in starch accumulation in tomato fruit [38]. Decreased alpha-galactosidase (spot R7) and beta-galactosidase (spot R26) abundance, which are crucial in catalyzing galactose to useful products utilized in cell wall metabolism [39-40], had adverse effects on A. mollis roots under tidal flooding stress. Meanwhile, beta-glucosidase (spot R10) and UDP-glucose 4-epimerase family protein (spot R35) were also decreased, indicating that the induction of polysaccharide catabolism and the interconversion of hexoses (glucose/galactose) were inhibited in A. mollis roots under tidal flooding stress [41-42].

3.3.3. Glycolysis, TCA cycle, and ethanol fermentation

Total soluble sugar and starch contents, as the carbon source, were stored and used for cell respiration [43]. Carbohydrates metabolism, especially glycolysis and the TCA cycle, mainly provides energy for plant growth and development [44]. Pyruvate produced via the glycolytic pathway into the TCA cycle and the consequent electrons is transferred along an electron transport chain and then return to the mitochondrial matrix via ATP synthase [45].
Most of the glycolysis and TCA cycle-related proteins were increased in *A. ilicifolius* tissues under tidal flooding stress, including triosephosphate isomerase (spot 34 and 46), phosphoglycerate kinase (spot 38, 49, and 52), enolase (spot 56, 68, and R30), malate dehydrogenase (spot 63, 64, and R37), and dihydrolipoamide dehydrogenase (spot 74). In *A. mollis*, the abundance of glycolysis and TCA cycle-related proteins mostly showed an increase in leaves and a decrease in the roots under tidal flooding stress. Similar to the proteomic data of a previous study [21], increased glycolysis and TCA cycle-related protein abundances contributed to the defense system of *A. marina* leaves under short-term inundation. The chemical energy conserved during the TCA cycle in form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) (redox equivalents with high-energy electrons) is converted to ATP, which plays a key role in the cell [45]. This process occurs in the inner mitochondrial membrane and is accomplished by ATP synthesis. Interestingly, we found that mitochondrial ATP synthase was decreased in *A. ilicifolius* leaves (spot 24, 32) but increased in *A. ilicifolius* roots (spot R16, R22, R26, R29), indicating that the requirement of ATP decreased accordingly. This implied that high levels of ATP synthase abundance might not be needed in *A. ilicifolius* leaves. Our study revealed the presence of vacuolar H+-ATPase (V-ATPase) subunit in *A. ilicifolius* tissues (spots 6, 43, 44, R10, R11, and R12). Increased V-ATPase abundance was helpful to maintain the cytosolic pH homeostasis and provide free energy to establish a proton motive force across membranes in *A. ilicifolius* tissues [46]. Physiological data showed the ATP level or energy charge of the *A. ilicifolius* tissues was increased to tolerance the tidal flooding stress (Fig. 5C, D).

Aldehyde dehydrogenase is involved in fermentation along with alcohol dehydrogenase (ADH) [47]. Due to the low efficiency of energy conservation under fermentation, an increased rate of glycolysis is required to sustain ATP production necessary for cell survival [48]. We found that the abundance of ADH (spot R36, R44, R45) increased with a concomitant reduction in glycolysis and TCA cycle-related proteins (spot R28, R37) in *A. mollis* roots under tidal flooding stress. Glycolysis converts glucose to pyruvate for the production of energy [45]. Thiamine diphosphate (spot R43), a co-factor of upregulated pyruvate, showed a decrease in *A. mollis* roots [49], indicating a reduction of pyruvate level. Physiological data showed the ATP level and energy charge of *A. mollis* roots became too low to sustain the basal metabolic requirement of roots (Fig. 5C, D).

### 3.4 Effect of tidal flooding on nutrient assimilation and protein metabolism of Acanthus species

Sugar metabolism provides sufficient energy to amino acid metabolism and intermediates from glycolysis can be utilized as precursors for the synthesis of amino acids [50]. In *A. ilicifolius* leaves and roots, many up-regulated proteins were associated with amino acid and protein metabolism (Fig. 5).

Nitrogen utilization is an important physiological activity in plant growth and development [48]. Increased abundances of glutamine synthetase (spot 53), glutamate-ammonia ligase family protein (spot 66, R34, R40), and glycine dehydrogenase (spot 75, 76) were considered to contribute to the conversion of ammonium generated from nitrate assimilation or photorespiration into amino acids in the cell of *A. ilicifolius* tissues. In *A. mollis* tissues, nitrogen metabolism-related enzymes, such as glutamine synthetase (spot 10, 44) and arginase (spot 56), showed an increase in leaves. Inhibited arginase activity led to the blockage of the nitrogen reutilization pathway in rice [51], indicating the promotion of nitrogen reutilization by increasing arginase abundance in *A. mollis* leaves.

S-adenosyl methionine synthetase (SAMS) catalyzes the biosynthesis of S-adenosyl-L-methionine (SAM) via the methionine cycle to participate in ethylene biosynthesis [50]. Overexpressed SAMS increased the ethylene level of *A. thaliana* [52], suggesting that the production of ethylene would be reduced in leaves and promoted in roots by tidal flooding stress. Meanwhile, MTA/SAH nucleosidase (spot 37), which showed an increase in *A. ilicifolius* leaves, promoted methionine recycling under tidal flooding stress [53]. Methionine is metabolized to homocysteine via SAM and SAH intermediates [54]. In the present study, tidal flooding stress influenced the abundance of SAH in *A. mollis*, which was decreased in the leaves (spot 50) but
increased in the roots (spot R29). In addition, decreased sulfite adenyltransferase (spot 48) and cysteine synthase (spot 34) abundances indicated adverse effects on producing cysteine from sulfate in A. mollis leaves [12, 55]. Overall, the sulfate assimilation pathway that feeds the biosynthesis of cysteine is suppressed in A. mollis leaves.

Plant tissue exposure to abiotic stress induces protein damage in cells. Therefore, plant broke down damaged proteins for cell survival [56]. Increased proteasome subunit alpha type (spot 17) and proteasome subunit beta type (spot 59) abundances were observed in A. ilicifolius under tidal flooding stress. Both DEPs are subunits of the 26S proteasome that was involved in the direct degradation of misfolded or oxidized proteins or via the ubiquitin-proteasomal pathway [57]. Furthermore, heat shock proteins (spots 10, 11, 26, 28, 29, and R13) mostly increased in A. ilicifolius under tidal flooding stress. These DEPs participate in plant fitness mainly by folding mature protein or degrading misfolded proteins [58]. Metabolism-related proteins in A. ilicifolius facilitate normal protein folding and guard the proteome against misfolding and aggregation under tidal flooding stress. In A. mollis, the abundance of proteasome subunit alpha type (spot 11, R14) and polyubiquitin (spot R13) were decreased but 26S protease regulatory subunit 6A homolog (spot R15) and 20S proteasome alpha subunit (spot R25) increased during the flooding stage.

In addition, protein synthesis essentially requires ribosomes, which play a distinct role in all living cells [59]. The biogenesis of 40S ribosomal subunits was enhanced by increasing RNA-binding protein NOB1 (spot 47) abundance in A. ilicifolius leaves. The tidal flooding stress decreased 60S ribosomal-related proteins (spot R1, R38) abundance, suggesting inhibition of protein synthesis in A. mollis roots. In A. ilicifolius roots, tidal flooding stress increased protein disulfide isomerase (spot 27) abundance, which is a molecular chaperone containing thioredoxin domains that help in the formation of disulfide bonds during protein folding [60].

### 3.5 Effect of tidal flooding on antioxidative defense system of Acanthus species

The antioxidative defense system has a stronger reactive oxygen scavenging capacity to mitigate oxidative damage under flooding stress [61]. Our data suggested that most of the antioxidant enzyme-related proteins, including l-ascorbate peroxidase (spot 36, R17), thioredoxin (spot R15, R31), monodehydroascorbate reductase (spot R20), and catalase (spot R36), were increased in A. ilicifolius under tidal flooding stress. The ascorbate-glutathione cycle together with 2-Cys peroxiredoxin is relevant systems in detoxifying reactive oxygen in stressed plants [62]. It has been reported that most of the enzymes (spot 2, R7, 24, and 49) involved in this process decrease in A. mollis.

In addition, annexin genes have been reported to have peroxidase activity [63]; it has been hypothesized that elevated abundance of annexin D5-like (spot 42) together with glutathione S-transferase (spot 60) modulate endogenous ROS levels in A. mollis leaves under tidal flooding stress. Moreover, glutathione S-transferase in protein regulation via S-glutathionylation, as a post-translational modification, have been reported in plants [64].

### 3.6 Effect of tidal flooding on transcription and signal transduction of Acanthus species

According to Ka/Ks ratios, 14-3-3 protein was a positive selection gene of Acanthus species, which plays an essential role in species survival in complex environments [15]. 14-3-3 protein is not only involved in signal transduction but also takes part in the regulation of carbon and nitrogen metabolism [65]. In soybean, the expression of 14-3-3 protein improved flooding resistance [66]. In the current study, the abundance of 14-3-3 protein increased in A. ilicifolius (spot 3, 14, R2, and R3) but decreased in A. mollis (spot 9, R4, and R8) under tidal flooding stress. As a secondary messenger to multiple signals response, inositol-tetrakisphosphate 1-kinase (spot R9) was decreased, indicating tidal flooding stress damage of the signal pathway of A. mollis roots.

Some redox-sensitive proteins involved in cellular structure, such as actin, profilin-4, tubulin α, and annexin D5-like, were found in Acanthus species. A previous study demonstrated that actin could be an important functional protein to response environmental factors [67]. Although actin filaments respond to UV-B radiation by influencing the process of mitosis, their dynamics are...
disrupted by salinity stress [65]. The actin abundance increased in A. ilicifolius leaves (spot 40, R21, R28) but decrease in A. mollis leaves (spot 67) to respond to tidal flooding stresses. Moreover, protein tubulin α (spot 22), which is also involved in mitosis, showed an increase in A. ilicifolius leaves. Therefore, although increased profilin-4 (spot R12) abundance stabilized the actin structure of A. mollis roots, we observed an increase in cell division-related proteins in A. ilicifolius leaves (spot 41, 42) and a decrease in A. mollis roots (spot R31).

4. Materials and Methods

4.1 Plant material and experimental setup

Experiment materials were obtained from vegetative propagation. The stems of A. ilicifolius (9-12 mm in diameter and 10-20 cm in length) were collected from the Zini mangrove forest (117°91 E, 24°45′ N), south of the Jiulong River Estuary, Fujian Province, China. The roots of A. mollis were collected from a mother plant that was planted in the greenhouse for one year. The explants of A. ilicifolius and A. mollis were placed in pots (19 cm in diameter and 20 cm in depth) with soil plus vermiculite in a ratio of 3:1. The growth of cuttings is shown in Supplementary Fig. S1. 1/8-strength Hoagland nutrient solution with rooting hormone powder was used to promote the growth of stems and adventitious roots. In the first two months, the cuttings were grown under controlled conditions: temperature (28 ± 2℃), weak light, and relative humidity (60 ± 5%). The pots were then transferred to a new condition with 1000 μmol·m⁻²·s⁻¹ light intensity, 12 h light period day⁻¹, and 28 ± 2℃ temperature. After growth for five months, uniform and healthy plants were selected for further analysis. The plants were randomly divided into two groups. The soil water content of the control group was kept at 65 ± 5% and regulated by the oven drying method. The pots were placed inside 50 L plastic containers maintaining a 1-2 cm water layer above the soil surface (Supplementary Fig. S2) and treated with flooding stress for 6 h per day. All the pots perforated at the bottom to ensure proper drainage. The dry weight of the leaves and roots samples were taken every two days. The extraction of protein and RNA, paraffin sectioning, and the measurement of energy (adenosine monophosphate (AMP), adenosine diphosphate (ADP), ATP), and hydrogen peroxide (H₂O₂) content were performed on the tenth day.

4.2 Determination of the dry weight

Five plants were randomly selected, washed with distilled water, and divided into two parts (leaves and roots). The oven-dried (at 70°C for 72 h) leaves and roots samples were measured for dry weight in grams (g) using an electric weight balance. The tidal flooding tolerance of each species was determined as the relative plant dry weight (dry weight under tidal flooding treatment divided by dry weight under control conditions, expressed as a percentage). Each measurement was repeated three times with five replications per treatment.

4.3 Anatomical features of leaves and roots

The leaf center and mature root were collected from A. ilicifolius and A. mollis for paraffin sectioning. The fresh tissues were fixed in formalin-acetic acid-alcohol (FAA) solution containing 70% ethanol, 5% acetic acid, and 4% paraformaldehyde for 72 h. Then, tissues were fixed and dehydrated in an ethanol series (50-100%). Tissues were cleared by xylene and embedded by paraffin (58°C). Paraffin-embedded tissue blocks were sectioned with 10 mm thickness, and then cross-sections were stained with 1% aqueous safranin and 0.5% fast green. Tissues sections were photographed under a light microscope (Leica DM4 P, Germany) to determine anatomical parameters.

4.4 Protein extraction and quantification
Protein extraction was according to the method described by He and Wang [68] with some modifications. After 10 days of tidal flooding treatment, 2-4 g of treated tissue from *A. ilicifolius* and *A. mollis* were ground in liquid nitrogen and extracted using the tricarboxylic acid (TCA)-acetone/phenol-methanol combined extraction method. The tissue powder was transferred to a centrifuge tube and precipitated by adding cold acetone solution containing 0.2% dithiothreitol (DTT), and then centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet was suspended in the 2× extraction buffer (20 mM Tris-HCl (pH 8.0), 250 mM sucrose, 10 mM ethylene glycol tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 2% β-mercaptoethanol) at 4°C for 15 min. Then the homogenate was vortexed at 4°C for 15 min by adding an equal volume of saturated phenol (pH 7.5). The homogenate was centrifuged at 12000 rpm for 30 min to obtain the upper phenol phase and then mixed with three volumes of ice-cold methanol (containing 0.1 M ammonium acetate). After overnight precipitation at -20°C, the mixture was centrifuged at 12000 rpm and discarded the supernatant. Then the pellet was washed once with ammonium acetate/methanol (0.1M) and thrice with acetone (containing 0.2% DTT). Protein quantification was performed using the Bradford [69] method with bicinchoninic acid as the standard. Each treatment was performed for three biological replications for quantitative analysis.

Gel strips (Immobiline Dry Strip, pH 4-7, 17 cm; Bio-Rad, Hercules, CA) and 12.5% polyacrylamide gels were used to separate the prepared samples in the first and second dimension, respectively. The first dimension was performed with an Etan IPG phor system (GE Healthcare Amersham Bioscience, Little Chalfont, UK). The second dimension was accomplished using Bio-Rad PROTEAN XL/PowerPac (Bio-rad, USA). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were stained with Coomassie Brilliant Blue R-250 and scanned with Uniscan M3600 (China) at 600 dpi. Gels were analyzed using PDQuest software (Version 8.0, Bio-Rad). The DEPs were obtained by pairwise comparison with a fold change ≥2.0 and a Student’s t-test (p<0.05). In-gel tryptic digestion and protein identification were performed according to the method of Liu et al. [70].

4.5 Determinations of AMP, ADP, ATP, and sugar content

The method of ATP, ADP, and AMP extraction was according to Chen et al. [71] with some modifications. The powder was obtained from 2 g tissue and then homogenized with 10 ml perchloric acid (0.6 mol·L⁻¹) at 4°C for 30 min. Then the extraction mixture was centrifuged at 6000 rpm for 20 min. The resulting supernatant (6 ml) was quickly neutralized to pH 6.5-6.8 with 1 mol·L⁻¹ potassium hydroxide solution and passed through a 0.22-μm syringe filter. The supernatant was diluted to 10 ml before measuring. Shimadzu® LC-20A Prominence high-performance liquid chromatography (HPLC) equipped with Syncronis C18 column (4.6 mm × 250 mm, Thermo Fisher Scientific) was used to measure ATP, ADP, and AMP contents. The mobile phase was 0.1% phosphoric acid and the flow rate was 0.8 ml·min⁻¹. The ultraviolet detection wavelength was 254 nm. The energy charge (EC) was calculated using the following formula: EC = [(ATP) + 1/2 (ADP)]/([ATP] + [ADP] + [AMP]). Data were expressed as means of the five replicates.

The starch and total soluble sugar contents were measured with the starch content kit and plant soluble sugar content test kit, respectively (Nanjing Jian Cheng Institute, Nanjing, China).

4.6 RNA extraction and gene expression analysis

Total RNA was extracted from 0.05 g of fresh tissues according to the manufacturer’s instructions using a MiniBEST plant RNA extraction kit (TaKaRa, Tokyo, Japan). The quality of RNA was examined by 1% (w/v) agarose gel electrophoresis. RNA was reverse-transcribed into cDNA using TaqMan™ reverse transcription reagents (Invitrogen, Life Technologies) and stored the cDNA at -80°C for further analysis. Primers were designed based on gene sequences listed in National Center for Biotechnology Information (NCBI) and transcriptome data from Yang et al. [15]. The primer sequences are provided in Supplemental Table S1.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using Bio-Rad CFX96 (USA) in 25 μL volumes, containing 12.5 μL 2× FastSYBR Mixture (CWbiotech,
China), 1 μL primers, 1 μL of the cDNA, and 10.5 μL RNase-free water. The amplification condition was initial denaturation (94°C for 10 min) followed by 40 cycles (30 s at 94°C, 30 s at 61°C, and 45 s at 72°C). The result was calculated using the $2^{-\Delta\Delta CT}$ method [72]. The relative gene expression was assayed along with β-actin as a reference gene and run in five biological repetitions.

4.7 Data analysis

The experimental data were evaluated with IBM SPSS Statistics for Mac (Version 23.0). The gels were analyzed using PDQuest software (Version 8.0, Bio-Rad). Principal Component Analysis (PCA) was performed using the OmicShare tools, a free online platform for data analysis (http://www.omicshare.com/tools). The classification of identified proteins was performed using the UniProt Knowledgebase (http://www.uniprot.org) and the NCBI (https://www.ncbi.nlm.nih.gov). The protein-protein interaction (PPI) network analysis was acquired using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 11.0 (https://string-db.org). Cytoscape software of the PPI network can visualize significant protein-protein associations [73]. The Cytoscape plugin (cytoHubba) was utilized to evaluate hub proteins from the PPI network by Matthews correlation coefficient (MCC) method [73]. We used the degree score to identify hub proteins.

5. Conclusions

Physiological and proteomic analyses have greatly enriched the current knowledge of flooding resistance in Acanthus species. A. ilicifolius performed better under tidal flooding stress, which was reflected in the integrity of the morphological structure, a high level of energy charge, and an increase in the ratio of soluble sugar to starch. A higher percentage of up-regulated proteins associated with carbon and energy metabolism were found in A. ilicifolius tissues under tidal flooding stress. However, the change in the root structure was not responsible for adaption to flooding conditions at the early stage and the maintenance of physiological homeostasis had higher demands for essential supply of energy. A. mollis leaves remained structurally intact even after tidal flooding stress, which might be due to partially enhanced ROS scavenging capacity and carbon and energy metabolism. The disruption of energy provision and flux balance in A. mollis roots demonstrates that maintenance of an energy balance under abiotic stress is critical for cell survival. As shown in Figure 10, we propose a working model to illustrate the detailed mechanism of A. ilicifolius and A. mollis under tidal flooding stress.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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