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BRIEF COMMUNICATION

Comparative proteomics of phloem exudates reveals long-distance signals potentially involved in *Litchi chinensis* flowering

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Abstract

It is well known that phloem is essential for transporting proteins as long-distance signals. In this study, a proteomic approach was carried out to identify proteins accumulated in phloem exudates at the pre-floral induction (PFId) stage, at the floral induction (FId) stage, and at the floral initiation (FIn) stage. As a result, 45 phloem exudate proteins were detected. Six proteins were found at all three stages. Sixteen proteins were specific to the PFId stage, 14 proteins were specific to the FId and FIn stages. From the enriched gene ontology (GO)-terms related to transport, signaling, hormone, and development, 12 transport-related, 5 signaling-related, 10 hormone-related, and 9 development-related proteins were identified. It was found that arginine glycine glycine repeats nuclear RNA binding protein A (RGGA) was specific to the FId stage whereas heat shock protein 90-7, plasma membrane intrinsic protein 1-4, and the homolog protein encoded by *At4g27190* were specific to the FIn stage. The relative abundances of sporulation protein F10D13.7 and ATP-dependent binding cassette transporter G family member 37 were higher at the FId stage than at the PFId stage, and that of enolase 2 was higher at the FIn stage than at the PFId stage, suggesting that these phloem exudate proteins might act as long-distance signals involved in the transition from vegetative growth to reproductive growth in litchi. This study contributes to the comparative proteomic analysis of plant phloem sap, which will provide insights into proteins involved in floral induction as well as inter-organ communication during development in litchi.

Additional key words: floral induction, floral initiation, GO-terms, litchi, pre-floral induction.

The plant xylem performs the important function for transport of water and nutrient, as well as for long distance-signals (Notaguchi and Okamoto 2015). It is well known that the phloem is essential for transport of small molecules, phytohormones, peptides/proteins, and coding/noncoding RNA (Lucas *et al.* 2013). These substances may play roles as long-distance signals produced from other tissues, and as media for crosstalk among organs, or between plants and environment.

Flowering is a transition from vegetative to the reproductive phase. In the model plant *Arabidopsis*, the floral stimulus is formed in the leaves. In the leaf tissues, CONSTANS (CO) activates *FLOWERING LOCUS T* (FT) (Samach *et al.* 2000), whose encoded protein is a long seeking florigen (Yang *et al.* 2007). The FT protein

migrates from leaves through phloem to the apical meristem to promote floral initiation (Corbesier *et al.* 2007). In other plants such as cucurbits and rice, the FT protein or its analogues are proved to be long-distance florigenic signals also transported in the phloem (Lin *et al.* 2007, Tamaki *et al.* 2007).

Litchi is an evergreen fruit tree commercially cultivated in southern Asia, South Africa, and Australia. Floral initiation in litchi is triggered by low temperatures and enhanced by drought in autumn and winter (Menzel and Simpson 1988, Chen and Huang 2005, Zhou *et al.* 2014). Litchi trees grown in controlled growth chambers with stably low temperature can produce big and high quality panicles (Lu *et al.* 2017). Interestingly, litchi trees grown under high temperature conditions with only few

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Abbreviations: ENO2 - enolase 2; GO - gene ontology; PFId - pre-floral induction; FId - floral induction; FIn - floral initiation; HSP - heat shock protein; PIP - plasma membrane intrinsic protein; RGGA - arginine glycine glycine repeats nuclear RNA binding protein A. *Acknowledgements:* The research results reported in this paper are funded by the National Natural Science Foundation of China (grants Nos. 31772249, 31572080), the National Litchi and Longan Research System (project No. CARS-32), the Innovation Team Project of the Department of Education of Guangdong Province (grant No. 2016KCXTD 011), and the Project of the Department of Education of Guangdong Province (2015KJGJHZ 016).

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leaves treated with low temperature still could produce flowers, though the panicles were small (Zhang *et al.* 2017), suggesting that the low temperature treated leaves can produce flowering signals, which can be transported to apical meristem to activate flowering.

In this study, a proteomics approach was carried out to identify proteins accumulated in phloem exudates before floral induction as the vegetative stage, at floral induction stage, and at floral initiation stage as the reproductive stages. We compared the proteins in the phloem exudates with the aim to identify potential long-distance signals produced in leaves that can be transported through phloem to regulate flowering.

Fourteen-year-old litchi (*Litchi chinensis* Sonn.) cv. anyuehong trees were grown in the experimental orchard of South China Agricultural University (a latitude of 23°9'40" N, a longitude of 113°21'18" E). When the fruits were harvested, trees were trimmed to allow three flushes. When the last flushes (terminal shoots) were mature, the terminal shoots with similar phenological stage were selected. The shoots were collected on 1st October as pre-floral induction (PFId) stage, 31st October as floral induction (FId) stage, and 10th November as floral initiation (FIn) stage in 2017. At the PFId stage, leaves of the terminal shoots were just mature and the buds were in dormant stage. At FId stage, the leaves became dark green and the buds were still in dormant stage. At the FIn stage, the buds sprouted and the panicle primordia could be found at the axil of the rudimentary leaves.

The terminal shoots in 6 cm length with two compound leaves were cut from the branches. Two leaflets in one compound leaves were kept. The cut shoots were re-cut in 20 mM Na₂-EDTA, and were placed in a microtube containing 2 cm³ of the Na₂-EDTA solution, incubated for 2 h. Then the solution was discarded. To remove all residual Na₂EDTA, the petioles of the cut shoots were washed with sterilized double-distilled water for 5 times. After that, the cut shoots were placed in a new microtube containing 1.5 cm³ of sterilized double-distilled water for exudate collection. All the cut shoots were incubated in boxes with humid atmosphere. The exudates were collected for 8 h according to the method described by Guelette *et al.* (2012). Ten microtubes of the exudates were pooled together and frozen in liquid nitrogen, and stored at -80 °C.

The phloem exudates were unfrozen at 4 °C and centrifuged at 12 000 g. To obtain pure protein, the phloem exudates were ultrafiltrated in 3 kD-ultrafiltration tube. The residue was washed using 100 mM ammonium bicarbonate for three times. To the washed residue (about 100 mm³), dithiothreitol was added at a final concentration of 50 mM, and incubated at 56 °C for 30 min. The iodoacetamide at a final concentration of 50 mM was added and the solution was incubated in the dark at room temperature for 30 min and then under irradiance for 5 min. The reacted solution was added with 5 times volume of acetone, kept at -20 °C overnight for protein deposition, and centrifuged at 12 000 g and 4 °C for 10 min. The residue was dried in cupboard wind, added with trypsin, dissolved in 50 mm³ of 100 mM ammonium bicarbonate. The mixture was

incubated at 37 °C for 16 h, and then lyophilized.

The samples were analyzed using *Easy-nLC* nanoflow HPLC system connected to *Orbitrap elite* mass spectrometer (*Thermo Fisher Scientific*, San Jose, CA, USA). Solution A contained 0.1 % (v/v) formic acid in double distilled water. Solution B contained 0.1 % (v/v) formic acid in acetonitrile. The column (*RP-C18* 0.15 × 150 mm, *Column Technology*, Fremont, CA, USA) was equilibrated using 95 % of solution A. Samples were loaded onto *Thermo Scientific EASY* column (two columns) by an autosampler and subjected to sequential separation by *Thermo Scientific EASY* trap column (150 μm × 1.5 cm, 5 μm particle size, 0.1 nm pore size, *C18*) and analytical column (75 μm × 25 cm, 5 μm particle size, 0.1 nm pore size, *C18*) using a segmented 1 h gradient from solvent A to 50 % solvent B for 50 min, followed by 50 - 100 % solvent B for 4 min, and then 100 % solvent B for 6 min.

The mass spectrometer was operated in positive ion mode, and MS spectra were acquired over a range of 300 - 2000 m/z. The resolving powers of the MS scan and MS/MS scan at 200 m/z for the *Orbitrap Elite* were set as 70 000 and 17 500, respectively. The top sixteen most intense signals in the acquired MS spectra were selected for further MS/MS analysis. The isolation window was 1 m/z, and ions were fragmented through higher energy collisional dissociation with normalized collision energies of 35 eV. The maximum ion injection time was set at 50 ms for the survey scan and 150 ms for the MS/MS scans, and the automatic gain control target values for full scan modes was set to 1.0 × 10⁻⁶ and for MS/MS was 5 × 10⁴. The dynamic exclusion duration was 30 s.

The raw files were analyzed using the *Proteome Discoverer v. 1.4* software (*Thermo Fisher Scientific*). Search for the fragmentation spectra was performed using the *MASCOT* search engine embedded in *Proteome Discoverer* against the self database *fasta*. The following search parameters were used: monoisotopic mass, trypsin as the cleavage enzyme, two missed cleavages, carbamidomethylation of cysteine as fixed modifications, and peptide charges of 2+, 3+, and 4+, and the oxidation of methionine, phospho (ST), GlyGly (K), acetyl (K), acetyl (N-term) was specified as variable modifications. The mass tolerance was set to 20 mg dm⁻³ for precursor ions and 0.1 Da for the fragment ions. The results were filtered based on a false discovery rate (FDR) of no more than 1 %. Gene ontology (*GO*) terms for the peptides were obtained from a *BLASTP* search against the *UniProtKB Swiss-Prot* database (available online: <http://www.uniprot.org/uniprot/>). Enriched *GO* terms were identified with the Fisher's exact test.

SDS-PAGE analysis and silver staining of the phloem exudates showed protein compositions at the three stages, suggesting the collected phloem exudates could be used to identify proteins by shotgun analysis. As a result of the shotgun analysis, we totally detected 45 proteins that were present in the phloem exudates (Table 1 Suppl.). Six proteins were found at the three stages, whereas 16 proteins were specific to the PFId stage, regarded as the vegetative stage, 14 proteins were at the FId and FIn stages, regarded as the reproductive stages (Fig. 1).

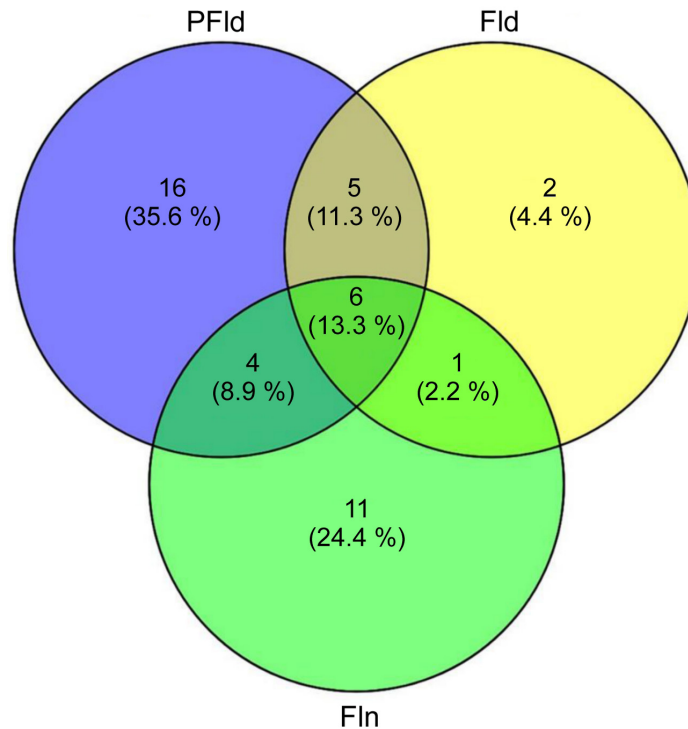


Fig. 1. A Venn diagram of the number of phloem exudate proteins at the pre-floral induction (PFId) stage, floral induction (FId) stage, and floral initiation (FIn) stage.

We then performed *GO*-term analysis. At the PFId stage, the top three hit enriched *GO*-term of the biological processes were the catabolic process, cellular catabolic process, and reactive oxygen species metabolic process. For cell component, the top hit enriched *GO*-terms were the cytoplasm, intracellular membrane-bound organelle, and membrane-bound organelle. For molecular function, the *GO*-terms were ion binding, metal ion binding, and cation binding. At the FId stage, the top hit enriched *GO*-terms of the biological processes were response to stimulus, response to stress, and reactive oxygen species. For cell component, the top hit enriched *GO*-terms were the cytoplasm and cytoplasmic parts. For molecular function, tetrapyrrole binding is the top hit enriched *GO*-term. At the FIn stage, the top hit enriched *GO*-terms were response to stress and catabolic processes in the biological process, cytoplasmic parts in the cell component, and copper ion binding in the molecular function.

From the enriched transport-related *GO*-terms including transport, RNA transport, auxin transport, transmembrane transport, regulation of transporter activity, protein transport, auxin polar transport, we identified 12 transport-related homologous proteins. As shown in Table 1 Suppl., they are L-ascorbate peroxidase (APX1), plastocyanin major isoform (DRT112), blue copper protein (BCB), ran-binding protein 1 homolog c (RANBP1C), peroxisomal (S)-2-hydroxy-acid oxidase (GLO5), ATP synthase subunit beta-3 (Atp3), triosphosphate isomerase (CTIMC), ATP synthase subunit alpha (ATPA), patellin-5 (PATL5), ABC transporter G family member 37 (ABCG37), probable aquaporin PIP1-4 (PIP1.4), and endoplasmic homolog (HSP90-7).

From the enriched signalling-related *GO*-terms including signal transduction, hormone-mediated signalling pathway, positive regulation of auxin mediated signalling pathway, we identified 5 signalling-related homologous proteins. They are peptidyl-prolyl *cis*-transisomerase (CYP19-1), GLO5, serine/threonine-protein kinase (TOR), RGG repeats nuclear RNA binding protein A (RGGA), and At4g27190 (Table 1 Suppl.).

From the enriched hormone-related *GO*-terms including response to hormone, response to abscisic acid, response to auxin, regulation of hormone content, brassinosteroid biosynthetic process, we identified 10 hormone-related homologous proteins. They are APX1, BCB, CTIMC, bifunctional enolase 2 (ENO2), ABCG37, TOR, chlorophyll *a-b* binding protein 2.2 (LHCB2.2), RGGA, acid phosphatase-like protein (ACP), and DRT112 (Table 1 Suppl.).

From the enriched development-related *GO*-terms including developmental process, reproductive system development, vegetative to reproductive phase transition of meristem, flower development, meristem development, regulation of meristem growth, we identified 9 hormone-related homologous proteins. They are APX1, peroxidase 53 (PER53), DRT112, acidic endochitinase (CHIB1), sporulation protein (F10D13.7), ABCG37, TOR, midasin (AtMDN1), HSP90-7 (Table 1 Suppl.).

From the above phloem exudate proteins related to transport, signalling, hormone, and development, we identified those specific to the FId and FIn stages or those with a higher relative abundance at the FId and FIn stage compared to those at the PFId stage. We found that RGGA was specific to the FId stage, while HSP90-7, PIP1.4,

and the homologous protein encoded by *At4g27190* were specific to the FIn stage. We also found that the relative abundance of sporulation protein F10D13.7 and ABCG37 was higher at the FId stage than at the PFId stage, and that amounts of ENO2 was higher at the FIn stage than at the PFId stage.

The plant phloem system provides an avenue for transport of proteins which may act as long distance-signals (Notaguchi and Okamoto 2015). Comparative proteomics analysis of phloem sap from resistant and susceptible rice cultivars has been carried out to investigate long-distance signalling (Du *et al.* 2015). Phloem exudates of *Arabidopsis thaliana* during the induction of systemic acquired resistance (SAR) with virulent or avirulent strains of *Pseudomonas syringae* has been performed to identify proteins contribute to the SAR response (Carella *et al.* 2016). Also, a study of phloem exudates collected from salt-stressed cucumber has been done to identify salt-responsive phloem proteins (Fan *et al.* 2015). These comparative proteomics studies are focused on biotic and abiotic stress responses.

Flowering, an important developmental stage for plants. Interestingly, it has been proven that the flowering signals are produced in leaves and transmitted through phloem system (Corbesier *et al.* 2007). In leaves, FT is activated by CO and then the FT protein in *Arabidopsis* and its analogue Hd3a in rice migrate through phloem to the apical meristem to promote floral initiation (Lin *et al.* 2007, Tamaki *et al.* 2007). Aki *et al.* (2008) performed a shotgun analysis of the proteome of phloem saps from rice, and found the presence of three TERMINAL FLOWER 1 proteins in phloem sap, suggesting that apart from the FT protein, other flowering-related proteins may exist in the phloem sap and act as long-distance signals. However, much less comparative proteomic studies on floral-inductive proteins in phloem exudates are reported than those on the stress-responsive proteins. Up till now, no publication has reported related studies on floral-inductive proteins in the litchi phloem exudates.

Litchi is an evergreen fruit tree requiring low temperature for floral induction (Chen and Huang 2005, Lu *et al.* 2017). However, litchi floral induction always conducts now under warm winter condition which is attributed to global warming, causing poor litchi flowering. It is important to reveal the genetic control of flowering so as to find methods to bypass chilling. One of these tasks is to find out the flowering-related proteins that may act as long-distance signals in the phloem saps. In our study, we performed shotgun analysis and detected 45 proteins that were present in the phloem exudates. From the phloem exudate proteins related to transport, signalling, hormone, and development, we further screened those specific to the FId and FIn stages, the reproductive stages, or those with a higher relative abundance at the FId and FIn stages compared to those at the PFId vegetative stage. As a result, we identified RGGA, HSP90-4, PIP1.4, a homologous protein encoded by *At4g27190*, F10D13.7, ABCG37, and ENO2.

The RGGA belonged to the *GO*-terms including the signal transduction (GO:0007165), hormone-mediated

signalling pathway (GO:0009755), in the response to hormone (GO:0009725), and response to abscisic acid (GO:0009737). It is a nucleus-localized RNA-binding protein, containing arginine-rich regions with several RGG repeats at their N- and C-termini (Landsberger *et al.* 2002). HSP90-7 is regarded to function in the protein transport (GO:0015031) and regulation of meristem growth (GO:0010075). Heat-shock protein 90 (HSP90) is a highly conserved molecular chaperone that is involved in modulating a multitude of cellular processes under both physiological and stress conditions (Chong *et al.* 2015). It can fold protein kinases and steroid hormone receptors (Richter and Buchner 2001, Young *et al.* 2001). HSP90-7 is an ER-localized HSP90 paralogue regarded as the HSP90B subfamily (Chen *et al.* 2006). The mutant in which a T-DNA is inserted in the promoter region of *HSP90-7*, has an enlarged dome-shaped shoot apical meristem (Ishiguro *et al.* 2002). The PIP1.4 belonged to the transmembrane transport (GO:0055085); it is a member of the plasma membrane intrinsic proteins. Constitutive overexpression of PIP1;4 promotes sap flow and water transport under cold stress (Jang *et al.* 2007). In this study, our result showed that RGGA, HSP90-7, and PIP1.4 were specific to the FId or FIn stages suggesting that these phloem exudate proteins might act as long-distance signals involved in the transition from vegetative growth to reproductive growth.

The sporulation protein F10D13.7 belonged to the *GO*-terms including the developmental process (GO:0032502), meristem development (GO:0048507), and regulation of meristem growth (GO:0010075). It plays a role in the regulation of root meristem cell division (Zhou *et al.* 2011). ABCG37 functions in a wide range of biological processes including transport, hormone, and development. It is an ATP-binding cassette (ABC)-type transporter (Ziegler *et al.* 2017); it transports the endogenous auxin precursor indole-3-butyric acid (Ruzicka *et al.* 2010). The ENO2 belonged to the *GO*-terms response to hormone (GO:0009725) and response to abscisic acid (GO:0009737). It is also called LOS2, and can bind to the promoter of *STZ/ZAT10* from *Arabidopsis*. It has a direct regulatory function in controlling *STZ/ZAT10* under low temperature (Lee *et al.* 2002). The low temperature is also needed for litchi floral induction. In the present study, we found that relative abundance of sporulation protein F10D13.7 and ABCG37 was higher at the FId stage than at the PFId stage, and that abundance of ENO2 was higher at the FIn stage than at the the PFId stage, suggesting that these phloem exudate proteins might act as long-distance signals involved in the transition from vegetative growth to reproductive growth.

The FT protein in *Arabidopsis* and its analogue Hd3a in rice are proved to be long-distance signals migrating through phloem to the apical meristem to promote floral initiation. However, we did not find the homologous protein FT in the phloem exudate of litchi. Aki *et al.* (2008) performed a shotgun analysis of the proteome of phloem sap from rice. They did not find the FT analogue Hd3a, but the presence of three TERMINAL FLOWER 1 proteins. It may be that the amount of this protein was too low to be determined. It may also be that other proteins but not

the FT might act as long-distance signals controlling floral induction in litchi. Further studies should be carried out to identify the florigen, and the functional studies should be performed for the identification of phloem exudate proteins in litchi.

In conclusion, our results provide the first insights into long-distance proteins involved in floral induction as well as inter-organ communication during development in litchi.

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