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Proceeding Paper

Identification and Characterization of PHT1 Transporters Family and Differential Expression Patterns in Control and Blindness Broccoli Plants †

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Abstract: Phosphorous is predominantly taken up by the plant from the soil as its inorganic form (Pi). This is energy-consuming process carried out by a family of high-affinity Pi transporters (PHT). The objective of the present study was the identification and characterization of the PHT1 Pi transporters family in *Brassica oleracea var. italica*, broccoli plants. A total of 31 PHT1 gene sequences were identified in broccoli plants and were fully characterized. In addition, RNA sequencing expression of control and blinded broccoli plants were carried out with different tissues in order to understand the implication of these transporters, PHT1, in broccoli blindness.

Keywords: phosphorus transport; broccoli genome; broccoli rna-seq; plant nutrition

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1. Introduction

Brassica oleracea var. italica also known as broccoli is an important crop worldwide mainly due to its beneficial nutritional characteristics displaying high concentrations of vitamins A, C, E and K and other metabolites that presents a wide range of beneficial bioactivities known as glucosinolates and its derivate molecules, isothiocianates and phenolic compounds [1]. However, when low amounts of available nutrients occurred, affected to the biological properties the plant. In this way, although, phosphorus is one of the most important nutrients, its influence in broccoli plant physiology has been poorly studied [2].

Phosphorus is normally taken up from soil by plants as its inorganic form (Pi). The uptake of Pi by plants is an energy consuming process. In this way we can find high and low-affinity transporters [3]. In the past, five high-affinity transporters families have been described in Arabidopsis thaliana (PHT1, PHT2, PHT3, PHT4, PHT5) [4]. In particular, the proteins belonging to PHT1 family have been shown to be essential under Pi deficiency. In this sense, the highly conserved PHT1 group is crucial for Pi uptake from the soil [5,6]. In Arabidopsis, a total of nine PHT1 transporters have been identified (PHT1;1-PHT1;9). AtPHT1;1 and AtPHT1;4 are important in Pi uptake in low and high availability Pi environments [7], while AtPHT1;8 and AtPHT1;9 play important roles only during phosphorous starvation [8]. Recently, a total of 49 PHT1 family members have been identified and characterized in Brassica napus, describing multiple transcriptional regulation that could refer to new roles of PHT1 genes in B. napus [9]. These findings should be researched as a possible interpolation in broccoli plants.

Therefore, the aim of the work was to determine the phosphorus transporters in broccoli and study their expression pattern in relation with a blindness phisiopathy (malfunction of the apical meristem) that is very common in brassica.

2. Material and Methods

2.1. Identification of Putative Broccoli PHT1 Transporters (PHT1)

The complete set of PHT1 transporters of broccoli (*Brassica oleracea var. italica*) were identified using blast protein algorithm against the broccoli (HDEM) proteome available in Genoscope date base (http://www.genoscope.cns.fr/plants, accessed on) and using as template sequences those from PTH1 proteins of *B. napus* identified by Li, Y et al. (2019).

2.2. Protein Charactesization, Sequence Analysis and Phylogentic Studies

Protein features such as amino acid length (No aa), molecular weight (Mw) were calculated with Expasy's ProtParam tool (https://web.expas/, accessed on). The transmembrane helices were predicted using TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/, accessed on). The subcellular location was predicted with two different prediction software: Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on) and an Eukaryotic protein subcellular localization predictor, DeepLoc tool (http://www.cbs.dtu.dk/services/DeepLoc/, accessed on).

Phylogenetic studies were performed with a tree construction with the sequences of PHT1 transporters form *Arabidopsis thaliana*, *Brassica napus* and *Brassica oleracea var. italica*. All the protein sequences were aligned with MUSCLE algorithm and to build the phylogenetic tree, a Neighbor Joining (NJ) algorithm with 1000 bootstrap replicates, a poisson model and pairwise deletion with the help of Mega X software [10].

2.3. RNA-seq Analysis

The quality of raw data (reads) were analyzed and mapped in broccoli genome using HISAT2 software. For differential expression analyses DESeq2 algorithm was used and normalized with rLog. All analyses were carried out in Galaxy web platform (https://usegalaxy.org/, accessed on).

2.4. Data Analysis

Statistical analyses were performed using the SPSS 25.0.0.1 software package. Statistical differences were calculated via Student's-*t*-Test and all parameters were determined at $p \leq 0.05$. The values presented are the means ± Standard errors (SE).

3. Results

3.1. Genome-Wide Identification of PHT1 Genes in Broccoli and Phyligenetic Analysis

A search of the whole genome of broccoli for PTH1 transporters revealed a total of 31 matches. Three of the sequences found were partial and other four sequences were non completed, almost full sequence, lacking the final part. Due to this, the partial sequences were excluded from the phylogenetic analysis and three constructions in order to not create interference in sequence alignments. The sequences were named according to chromosome location from lower to higher numbers.

For the phylogenetic analyses the protein sequences of Pi transporters found in broccoli proteome were aligned against those from *B. napus* and *A. thaliana*. Arabidopsis PHT1 transporters are divided into nine subfamilies, forming nine groups in the phylogenetic three (Figure 1).

3.2. Chromosmal Location and Protein Features and Subcellular Location Predictions

When focus in gene location, all the genes were widely distributed in all chromosomes, except in chromosome 1, where no PHT1 like gene were found. On the other hand, the chromosome with highest number of PHT1 genes were chromosome 9 followed by chromosomes 2 and 4. When focus in protein features, the complete sequences found were in the range of 509 to 557 amino acid length and molecular weight between 56–59 kDa. Transmembrane transporters proteins have normally transmembrane domains formed by

α-helixes forming the pore, in this case, a total of 12 α-helixes were predicted to form the PHT1 proteins. 3D structures were also analyzed and almost all presented 12 transmembrane motifs (data not shown). Finally, subcellular location predictions determined that the main location of almost all the proteins were plasma membrane when analyzed with Plant-m-Ploc. On the other hand, *deep loc* analysis also indicated that some proteins could be located also in the endoplasmic reticulum and vacuoles (tonoplast).

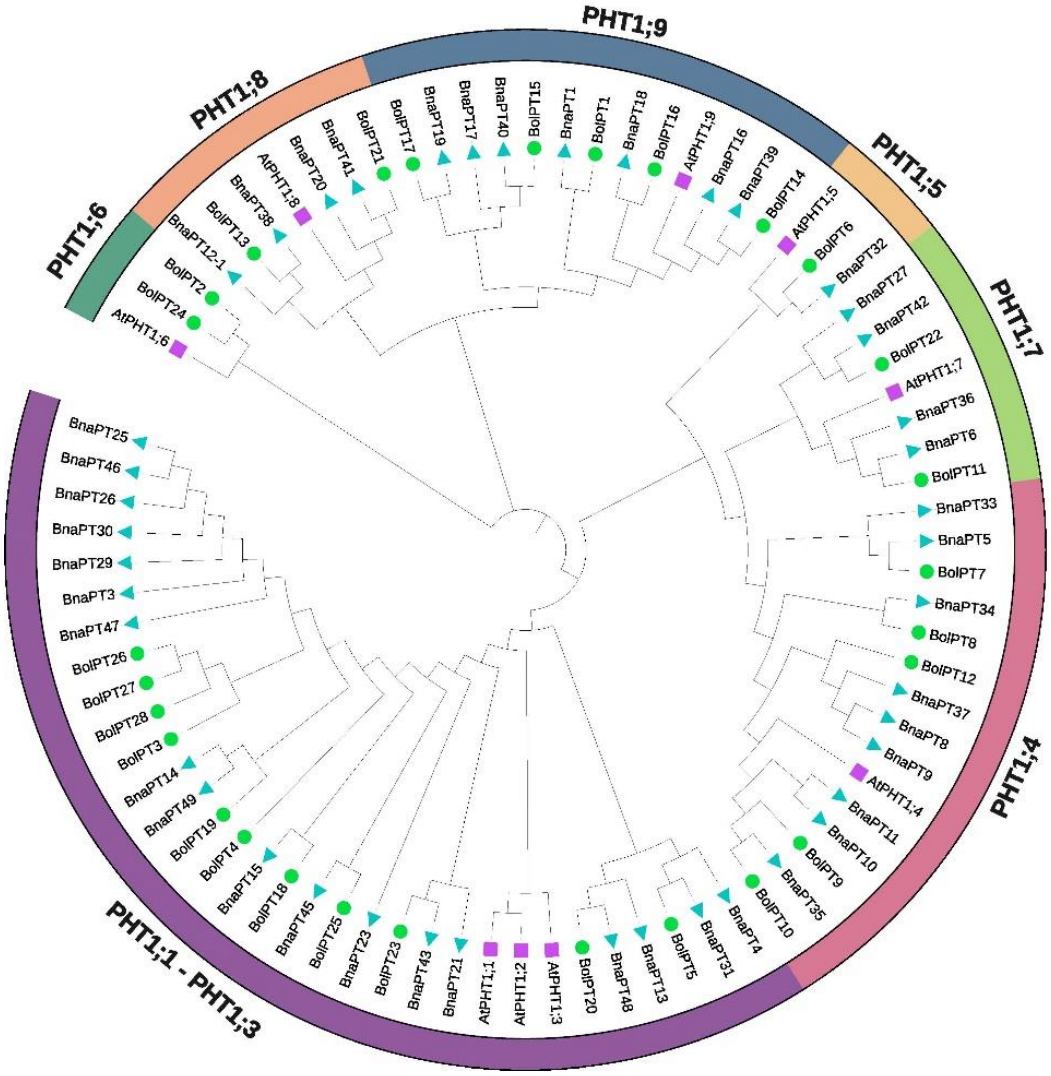

Figure 1. Phylogenetic analysis of PTH1 proteins of *B. oleracea var. italica* (circles), *A. thaliana* (squares) and *B. napus* (triangles). Muscle were used to align protein sequences and the NJ method (with 1000 bootstrap replication) to build the tree, all with MEGA X. The different PTH1 subfamilies are also represented.

3.3. RNA-seq Analysis, Expression Studies

Different analyses of expression were carried out (Figure 2). Expression of PHT1 family genes were compared on different tissues, root and leaves (Figure 2a) showing that the most expressed gene in leaves was BolPT1 showing almost fold expression when compared with root. Meanwhile, BolPT6, BolPT7, BolPT9, BolPT10, BolPT11, BolPT16, BolPT17 and BolPT27 genes showed no statistical differences in expression between the two types of tissues. Alternatively, BolPT14, BolPT18, BolPT21, BolPT22, BolPT26 and BolPT29 showed higher expression levels in root when compared with leaves. Some of the PTH1 genes showed no expression neither in roots and leaves so were not included on the graphical representation (Figure 2).

Differences in expression between control plants and plant with blindness were also measured (Figure 2b). On one hand, when analyzed gene expression, statistical differences in expression were found in predominantly in leaves when compared control with blindness, highlighting PT28 and PT8 that showed a repressed expression in the case of blindness broccoli plants leaves. However, BolPT11 and BolPT12 presented higher expression levels in blindness plants leaves when compared with control plants.

On the other hand, only PT28 showed to be statistically different being over expressed in blindness plants roots, almost two fold, when compared with control plants.

Table 1. List of 31 PHT1 transporters genes found in broccoli. Column identifiers (Gene name, ID) chromosome location (Chr loc), protein amino acid length (No aa), molecular weight (Mw), number of transmembrane domains (Mw) and cellular location. Numbers 1 and 2 are related with the algorithm used to make the prediction, 1: deep loc; 2: Plant m-Ploc. Symbols † indicate partial sequences and * symbols show not completed sequences.

Name	Gene ID	Chr loc	No aa	Mw (g/mol)	TMMH [a]	Cellular Location
BolPT1	BolC2t09393H	2	538	59,106, 69	12	E.R. [1], P.M. [2]
BolPT2	BolC6t40089H	6	537	59,136, 52	12	E.R. [1], P.M. [2]
BolPT3	BolC6t38329H	6	551	60,907, 72	12	E.R. [1], P.M. [2]
BolPT4	BolC6t40088H	6	538	59,064, 55	12	E.R. [1], P.M. [2]
BolPT5	BolC8t49671H	8	557	61,462, 94	12	E.R. [1], P.M. [2]
BolPT6	BolC2t10437H	2	517	56,464, 16	11	P.M. [1,2]
BolPT7 *	BolC9t55444H	9	444	48,159, 46	10	P.M. [1,2]
BolPT8	BolC9t55475H	9	509	55,452, 65	11	P.M. [2], Vacuole [1]
BolPT9	BolC8t50596H	8	531	58,264, 55	11	P.M. [2], Vacuole [1]
BolPT10	BolC4t22465H	4	534	58,586, 23	11	P.M. [2], Vacuole [1]
BolPT11	BolC4t28201H	4	529	57,910, 35	11	P.M. [2], Vacuole [1]
BolPT12 *	BolC4t22466H	4	417	46,203, 77	8	P.M. [2], Vacuole [1]
BolPT13	BolC2t10439H	2	521	57,251, 2	11	P.M. [2], Vacuole [1]
BolPT14	BolC7t43120H	7	521	57,250, 22	11	P.M. [2], Vacuole [1]
BolPT15	BolC9t55490H	9	521	57,222, 17	11	P.M. [2], Vacuole [1]
BolPT16	BolC9t55477H	9	521	57,220, 24	11	P.M. [2], Vacuole [1]
BolPT17	BolC9t55480H	9	521	57,204, 24	11	P.M. [2], Vacuole [1]
BolPT18	BolC2t10445H	2	535	58,692, 65	11	P.M. [1,2]
BolPT19	BolC7t43121H	7	535	58,392, 07	11	P.M. [1,2]
BolPT20	BolC3t14963H	3	535	58,656, 36	12	P.M. [1,2]
BolPT21	BolC4t25357H	4	540	59,244, 65	11	P.M. [1,2]
BolPT22	BolC4t22464H	4	533	58,692, 05	12	P.M. [1,2]
BolPT29 †	BolC4t27979H	4	112	11,940, 99	1	P.M. [1,2]
BolPT23	BolC5t30648H	5	542	59,950, 3	12	P.M. [1,2]
BolPT24 *	BolC7t43115H	7	464	50,582, 37	11	P.M. [2], Vacuole [1]
BolPT25 *	BolC2t10440H	2	450	48,991, 7	11	P.M. [2], Vacuole [1]
BolPT26	BolC6t40092H	6	506	56,402, 57	12	P.M. [1,2]
BolPT27	BolC9t55476H	9	521	57,381, 33	11	P.M. [1,2]
BolPT30 †	BolC9t55487H	9	176	19,122, 03	4	P.M. [2], Vacuole [1]
BolPT31 †	BolC2t10438H	2	148	15,945, 88	3	P.M. [2], Vacuole [1]
BolPT28	BolC3t14547H	3	546	59,316, 62	10	P.M. [2], Vacuole [1]

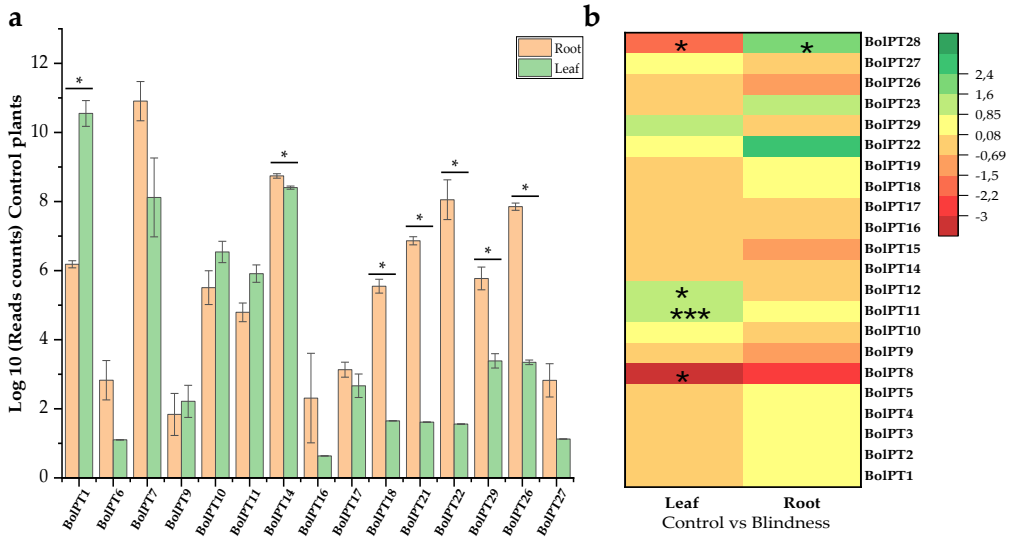

Figure 2. Analysis of RNA sequencing expression of PHT1 genes from broccoli plants in leafs and roots. (**a**) Expression of several PTH1 genes of control broccoli plants (SE errors bars); (**b**) Heat map representing fold change of PHT1 genes comparing broccoli control plants versus blindness broccoli plants in leaf and roots. Statistical differences were calculated with Student *t*-Test and are shown with asterisk (* $p < 0.05$; ** $p < 0.005$ and *** $p < 0.0005$).

4. Discussion

Nowadays the information available of complete genome sequencing of all king of living organisms is increasing due to the amelioration of the techniques and the continuous price drop of sequence cost per base [11]. Therefore, the increase of complete genome sequences requires an analysis and characterization of functional sequences like genes.

In the case of the broccoli plants, a total of 31 PHT genes were identified, in contrast with the 41 genes identified in B. napus [9] and 9 genes present in A. thaliana [8]. These findings reveal that PHT1 family is heterogeneous and its presence, number and functionalities depends on the plant species and family. Furthermore, it is noteworthy the high copy number variation of PHT1 family genes between Brassica species.

Additionally, the protein features such as molecular weight, transmembrane helix domains and cellular location prediction showed similarities to those of B. napus and A. thaliana [9,12]. Moreover, all PHT1 transporters protein share the same three-dimensional structure. Analysis predicts that these transporters family are characterized by having 12 membrane-spanning domains (data not shown) [13].

The PHT1 transporters are one of the most studied plants phosphorous transporters [6]. In particular, these proteins are responsible of Pi uptake from soil [14]. In this sense, our expression analysis revealed and overall higher expression of PTH1 transporters in roots (Figure 2a). Despite this, BolPT1 showed higher expression levels in leaf when compared root expression. These findings reveal a different role in the case of broccoli plants when compared with the role attributed in A. thaliana. The BolPT1 protein is included in PHT1;9 (Figure 1) and this subfamily have been shown to be involved in Pi uptake by roots in Pi starved plants in Arabidopsis [8]. Further analyses should be carry out in order to determine the role of BolPT1 in broccoli leaves.

Moreover, when compared the expression of PHT1 transporters of control and blindness plants (Figure 2b) we found that mostly changes occur on leaf gene expression. This could be result of blindness being a meristematic tissue disease resulting in major changes in gene expression of leaves with apparently no affected roots.

5. Conclusions

In this work the sequences of PHT1 genes have been assessed in broccoli. The analysis with related plant species was useful to classify the genes by families. This analysis together with RNA-seq carried out in roots and leaves of control and blindness broccoli plants show an overview of PHT1 transporters family. Also, the results showed a different expression in some of the transporters mainly (BolPT8, BolPT28, BolPT11 and BolPT12). Therefore, the involvement of this transporters in broccoli blindness opens a new line of research that points to the importance of phosphorus nutrition in the appearance of physiopathies as blindness.

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