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Genes involved in stress signals: the CBLs-CIPKs network in cold tolerant *Solanum commersonii*

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Abstract

Several studies revealed the important contribution of *calcineurin B-like* (*CBLs*) and *CBL-interacting kinase* (*CIPKs*) genes in transmitting stress signals in plants. Taking advantage from the genome sequences of the cultivated potato *Solanum tuberosum* and its wild relatives *S. commersonii* and *S. chacoense*, we identified for the first time 10 *CBLs* and 26 *CIPKs* genes in each species. The *CBLs* and *CIPKs* derived from tandem duplications indicate that these gene families in potato mainly arise through amplification mechanisms. Once annotated, we compared the *par excellence* model of *Arabidopsis thaliana* with *S. commersonii,* the potato model species for studying cold tolerance. We found that four ScCBL proteins (ScCBL1, ScCBL4a, ScCBL4b, and ScCBL9) started with a conserved N-myristoylation motif (MGXXXS/T), which might function in membrane targeting of the CBLs-CIPKs complex. Additionally, expression analyses of *S. commersonii CBL* and *CIPK* genes based on RNAseq revealed diverse expression patterns following various abiotic and biotic stresses and in the four tissues analyzed (flowers, leaf, roots, and tubers). Data also suggest that the *ScCBLs-ScCIPKs* complex may be more responsive to abiotic rather than biotic stimuli. Overall, the results described in the present work will be useful for future investigations and for functional characterization of individual *CBLs* and *CIPKs* in *Solanum*.

Additional key words: abiotic and biotic stresses, regulation of gene expression, signal transduction, wild potato.

Introduction

To adapt to various environmental stimuli, plants have evolved a series of complex signal transduction mechanisms. These pathways activate when plants sense external stimuli through plasma membrane, triggering a surge of Ca^{2+} into the cytosol. The up-take is perceived by calcium-binding proteins, including Ca^{2+}-dependent kinases (CDPKs), calcineurin B-like (CBLs), and CBL interacting kinases (CIPKs). These function as sensor molecules receiving signals from cellular calcium and transmitting them to downstream pathways (Li *et al.* 2016). Depending on the nature of the response domain (either a kinase or transcription regulation domain), Ca^{2+} sensor proteins are classified into sensor relays or sensor responders. Among sensor relay proteins, CBLs are thought to play a central role in the Ca^{2+}-signaling pathway in plants and some protozoans (Tuteja *et al.* 2007), binding Ca^{2+} with their four conserved EF hand motifs (Kudla *et al.* 1999). Once bounded Ca^{2+}, CBLs specifically target a group of sucrose non-fermenting 1-related serine/threonine proteins (SNF1), namely CIPKs, to transduce the perceived calcium signal (Shi *et al.* 2000). Commonly, CIPK proteins consist of a conserved N-terminal kinase domain and a C-terminal regulatory domain, which is separated from the former by a variable junction domain (Cheng *et al.* 2002). In particular, Ca^{2+}-bound-CBLs interact with and activate the catalytic domain of targeting CIPKs through conserved motifs (namely NAF or FISL). Among all the interactions between sensor relay proteins and sensor responders, the CBL-CIPK network serves as an essential signaling network regulating plant responses to low temperatures, salinity, K^+ deficiency, excess of Mg^{2+}, and drought (Yang *et al.* 2008, Held *et al.* 2011, Huertas *et al.* 2012). It also modulates growth and development, absorption and/or transport of nitrate, ammonium, and iron, sustaining H^+ homeostasis, and transduction of reactive oxygen species signals in plants (Pandey *et al.* 2015, Thoday-Kennedy *et al.* 2015, Mao *et al.* 2016). Therefore, the identification and comprehensive study of these sensors may provide useful insights for plant breeding.

Nowadays, the identification of genes, transcripts,

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Abbreviations: aa - aminoacids; CBL - calcineurin B-like; CIPK - CBL-interacting kinase; *PGGD* - plant genome duplication database; RPKM - reads per kilobase of transcript per million mapped reads.

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and proteins useful for breeding involves the use of new advanced bioinformatics tools. Among them, high-throughput genome sequencing efforts have dramatically increased knowledge of and ability to discover and characterize gene families in plants. For example, taking advantage from the genome sequence of *Solanum commersonii* (Aversano *et al.* 2015), we have disclosed and characterized several gene families (D'Amelia *et al.* 2014, 2018, Aversano *et al.* 2017, Esposito *et al.* 2018a,b). So far, bioinformatics has also been successfully used in the identification of both CBLs and CIPKs in different species. For example, Mohanta *et al.* (2015) described the *CBLs* gene family in 38 different plant species (among them *Glycine max*, *Arabidopsis thaliana*, *Cucumis sativus*, *S. lycopersicum*, and *S. tuberosum*) whereas many *CIPKs* genes have been described in *Arabidopsis*, *Brachypodium distachyon*, rice, and maize (Kolukisaoglu *et al.* 2004, Chen *et al.* 2011, Wang *et al.* 2015).

Potato (*Solanum tuberosum* L.) is the third most important crop in the world. It is sensitive to a wide range of biotic and abiotic stresses leading to losses in production almost every year. Its susceptibility might be due to the domestication during which the cultivated forms have lost critical genes important for adaption to the environmental challenges, as already reported in *G. max* (Lam *et al.* 2010). For these reasons, a number of wild tuber-bearing *Solanum* species represent valuable genetic resources to improve resistance traits (Machida-Hirano *et al.* 2015, Mori *et al.* 2015). Among wild potatoes, *S. commersonii* is not only the most tolerant to low temperatures, but also possesses a high capacity to cold acclimation (*i.e.*, ability to increase cold tolerance after exposure to low, non-freezing temperatures) (Palta and Simon, 1993) and resistances to root knot nematode, soft rot and blackleg, bacterial and verticillium wilt, *Potato virus X*, tobacco etch virus, common scab, and late blight (Hanneman and Bamberg 1986, Hawkes 1990, Micheletto *et al.* 2000, Carputo *et al*. 2009, 2013). Since its genome sequence is available (Aversano *et al.* 2015), it has become a model species for studying plant tolerance to cold as well as other stresses. Despite extensive studies of the *CBL-CIPK* network in other important crops, there are no reports regarding a comprehensive and detailed analysis of these gene families in potatoes. Also, how *CBL-CIPK* evolved in wild tuber-bearing potato species is still unknown. In this study, we exploited the genome sequence of three potato species (the cultivated *S. tuberosum* and its wild relatives *S. commersonii* and *S. chacoense*) to identify genes belonging to both *CBL* and *CIPK* families. Once genes were identified, we compared our model species for studying plant tolerance traits (*S. commersonii*) with the "par excellence" model *A. thaliana.* Further aim was to analyze *ScCBLs* and *ScCIPKs* gene families and their phylogenetic relationship, gene structure, protein motifs, gene duplications, and divergence.

Materials and methods

***In silico* identification of *CBL* and *CIPK* genes:** *Solanum commersonii, S. tuberosum*, and *S. chacoense CBL* and *CIPK* genes were identified through the following steps: *1*) all well-characterized *Arabidopsis CBLs* and *CIPKs* were downloaded from *TAIR* (https://www.arabidopsis.org/index.jsp) database and aligned through *ClustalW* (Larkin *et al.* 2007); *2*) the alignment file was used to build a similarity matrix using *hmmr v. 3.2.1* (*hmmer.org*); *3*) the resulted matrix was run as a bait against the *S. commersonii, S. tuberosum*, and *S. chacoense* genomes with e-values $< 1e^{-5}$; and *4*) only genes with an e-value $< 10^{-5}$ and an identity > 55 % were regarded as *CBL* and *CIPK* genes. The identified CBLs and CIPKs were further confirmed manually checking the specific domains using the *NCBI* search domain online tool (Marchler-Bauer *et al.* 2015). Names were assigned based on their orthologs in *Arabidopsis* using bootstrap replicates of the maximum likelihood phylogenetic tree (values higher than 50). In particular, only one-to-one orthologs were considered when genes allocated on the same clade in the phylogenetic tree with *Arabidopsis*. Otherwise, the names of the CBL and CIPK were assigned using the *prefix-like* and/or *prefix-specific.*

Phylogenetic analysis of gene structure and conserved motifs: All CBL and CIPK protein sequences and those of *Arabidopsis* were aligned using *muscle* (*https://www.ebi.ac.uk/Tools/msa/muscle/*). Then, *MEGA7* (Kumar *et al.* 2015) was first used to establish the best-fit model of evolution through the option "Find best DNA/protein models" implemented in the program. Finally, a maximum likelihood tree with the appropriate options was constructed with 1 000 bootstrap replicates. The gene structure of *S. commersonii CBLs* and *CIPKs* was analyzed using the gene structure display server (*GSDS 2.0*, http://gsds.cbi.pku.edu.cn/). Predictions of motifs were generated using Multiple Em for Motif Elicitation (*MEME*) program (http://meme-suite.org/tools/meme), with an optimum width of motifs ranging from 6 to 50, the maximum number of motifs set to 18, and default values for other parameters. Palmitoylation sites were predicted by *CSS-Palm 3.0* (http://csspalm.biocuckoo.org/) whereas myristoylation sites by *Myristoylator* (http://web.expasy.org/myristoylator/).

Gene duplications and evolutionary analysis: Tandem duplicated genes were defined as an array of two or more genes located on the same chromosome/scaffold and found within a 100 kb genomic window (Zhu *et al.* 2016). The locus search tool at the plant genome duplication database *PGGD* (http://chibba.agtec.uga.edu/duplication/) was used to determine if *CIPK* and *CBL* genes existed within genomic duplication blocks whereas the *CoGe* platform (https://genomevolution.org/coge/) was used to confirm tandem duplications.

Public RNA-seq based expression analysis: We used *fastq* data related to four tissues (flower, leaf, tuber, and stolon) and deposited in the sequence read archive (*SRA*) under study *SRP050412* to analyze the expression profiles of *ScCBLs* and *ScCIPKs*. Unmapped reads were considered

for the next analysis and were mapped against the *S. tuberosum* genome (*ITAG* annotation *v. 1*). The *Binary Alignment Map* files were then analyzed using the *Cufflinks-Cuffquant* software (*v. 2.2.1*) to assemble the aligned reads and to access transcriptome complexity. Expression values for each gene were estimated based on reads per kilobase of transcript per million mapped reads (RPKM) using the default options. Transcriptions of all *ScCBL* and *ScCIPK* were also characterized in potato leaves from plants that were subjected to various treatments including heat stress (35 °C for 24 h), salt stress (50 mM NaCl for 24 h), osmotic stress (260 μM mannitol for 24 h), 50 mM β-aminobutyric acid, 50 mMbenzothiadiazole, 10 mM indole-3-acetic acid, 50 mM abscisic acid, 50 mM gibberellic acid, and 50 mM 6-benzylaminopurine. In particular, data were retrieved from the public *S. tuberosum* database available in literature (http://solanaceae.plantbiology.msu.edu). The RPKM values of *S. commersonii* orthologs in control and stress conditions were collected.

Network and protein interaction of *S. commersonii* CBLs and CIPKs: For the protein interaction network, high confidence experimental data of interacting CBL and CIPK proteins in *Arabidopsis* were constructed using *STRING* (http://string-db.org/) with a confidence value > 0.7. The ortholog proteins of the determined interacting *S. tuberosum* proteins were identified in *S. commersonii* by reciprocal *BLASTP* analysis.

Results

Ten non-redundant *CBL* and 26 *CIPK* genes were identified in *S. commersonii*, *S. tuberosum*, and *S. chacoense* (Table 1) suggesting that their loci were substantially conserved after their evolutionary divergence. The multiple aminoacids (aa) sequence alignment of CBL proteins along with AtCBLs indicate that the general structures of CBLs were highly conserved with all CBLs having EF hands similar to those of AtCBLs (data not shown). Similarly, the multiple sequence alignments of CIPK proteins show that all members contain an N-terminal catalytic kinase domain and a C-terminal regulatory domain, which are necessary and very important for protein-protein interactions (data not shown). Once identified, we annotated and named each CBL and CIPK according to their phylogeny and protein sequence similarities with those of *Arabidopsis* (Fig. 1). In this way, closely-related orthologous and paralogous pairs of CBLs and CIPKs were identified based also on a high bootstrap value. As far as *S. commersonii CBLs* are concerned, two genes clustering on the same clade with *AtCBL4* were identified as *ScCBL4a* (on scaffold27252) and *ScCBL4b* (on scaffold3852). Two paralogs of *CBL4* were also identified in *S. tuberosum* (PGSC003DMP400016074 and PGSC0003DMP400034042) and *S. chacoense* (g9425.t1 and g21063.t1) (Fig. 1*A*) suggesting that *CBL4* might have been duplicated before the divergence of the potato species. Similarly to *ScCBL4*, genemark_C2860222_abinit_gene_0_8 and maker_scaffold12242_snap_gene_0_48 clustered with *AtCBL8*. Hence, we named them *ScCBL8a* and *ScCBL8b,* respectively. Interestingly, two paralogs of *CBL8* were found only in *S. commersonii* whereas one member was found in *S. tubersoum* (PGSC0003DMP400035601) and in *S. chacoense* (g6456.t1). By contrast, two paralogs of *CBL5* were identified only in the cultivated *S. tubersoum* but not in its wild relatives (Fig. 1*A*). The remaining *CBLs* were named using the *prefix-like* based on clustering with *Arabidopsis.* As far as *CIPKs* are concerned, *CIPK6*, *CIPK11*, *CIPK14*, and *CIPK20* had two paralogs in all the species analyzed (Fig. 1*B*). The *CIPK3* also underwent duplication events. Indeed, two copies were found in *S. commersonii* and *S. chacoense* whereas *S. tuberosum* harbored only one copy. The remaining *CIPKs* were identified as single orthologs (Fig. 1*B*). Furthermore, four *CIPKs* from *S. tuberosum,* three from *S. commersonii* and two from *S. chacoense* were named using the *prefix-specific* because they did not cluster with *AtCIPKs*. Detailed information regarding domain composition and the number of orthologs and paralogs of all species are reported in Table 1 Suppl., Figs 1 Suppl. and 2 Suppl.

Once genes were identified, we surveyed duplication events (data not shown). Phylogenetic results were confirmed through this analysis. Indeed, two tandem duplications were detected in *S. tuberosum* and *S. commersonii CBLs* whereas one was identified in *S. chacoense* (data not shown). The gene pairs include *CBL4a/CBL4b* in all the three genomes analyzed here, *CBL5a/CBL5b* in *S. tuberosum* and *CBL8a/CBL8b* in *S. commersonii.* In contrast, no segmental duplications of *CBL* genes were detected. A higher number of tandem duplications events (four events in *S. tuberosum* and five in *S. commersonii* and *S. chacoense*) were detected among *CIPKs.* The gene pairs include *CIPK6a/CIPK6b, CIPK11a/CIPK11b*, and *CIPK20a/CIPK20b* in all the species analyzed whereas *CIPK3a/CIPK3b* were found duplicated in *S. commersonii* and *S. chacoense* but not in *S. tuberosum*.

Intron/exon organization of all *CBLs* and *CIPKs* were analyzed to further investigate their structural features. In the case of *CBLs*, the number of exons among the three species was generally conserved. Indeed, *CBL1-like*, *CBL3-like, CBL4a*, *CBL8a*, *CBL9-like*, and *CBL10* harbored the same number of exons in *S. commersonii, S. tuberosum*, and *S. chacoense,* respectively. By contrast, differences were identified in *CBL4b* and *CBL8b* (Fig. 2 Suppl.). For example, *CBL4b* harbor 16 exons in *S. commersonii* and 8 in *S. tuberosum* and *S. chacoense* whereas *CBL8b* had 12 exons in *S. commersonii,* 7 in *S. tuberosum*, and 8 in *S. chacoense.* The *CBL* gene exon structure validated the protein length (Table 1). Indeed, *ScCBL4b* (16 exons) encoded for a peptide of 426 aa whereas the orthologs in *S. tuberosum* and *S. chacoense* (8 exons) was 215 aa in length. Also, the product of *ScCBL8b* was longer than *StCBL8b* and *SchacoCBL8* (320 aa in *S. commersonii,* 196 aa in *S. tuberosum*, and 221 aa in *S. chacoense*) reflecting differences in their gene structure. By contrast, members belonging to the *ScCIPK* family clustered into two different sub-groups (an intron-rich clade and an intron-poor clade) (data not shown). In particular, 18 *CIPKs* (*CIPK4-like*,

CIPK5-like, *CIPK6a*, *CIPK6b*, *CIPK7-like*, *CIPK11a*, *CIPK11b*, *CIPK14a*, *CIPK14b*, *CIPK16-like*, *CIPK20a*, *CIPK20b*, *CIPK22*, *CIPK24-like*, *CIPK2-like*, *CIPK_specific1*, *ScCIPK_specific2*, *ScCIPK_specific3*) belong to the former group whereas eight *ScCIPKs* (*CIPK17-like*, *CIPK1-like*, *CIPK23*, *CIPK24*, *CIPK3a*, *CIPK3b*, *CIPK8*, and *CIPK9*) belong to the latter. All motifs found were highly conserved in all species (data not shown). Similar to AtCBLs, CBLs possessed a conserved serine residue in the PFPF motif, which is phosphorylated by CIPKs (Du *et al.* 2011) whereas all CIPKs contained conserved N-terminal catalytic kinase and C-terminal regulatory domain. Furthermore, searching for other possible motifs that could be functionally important in fulfilling CBL roles, we found that CBL1-*like*, CBL4a, CBL4b and CBL9 harbored a conserved N-myristoylation motif (MGXXXS/T) that

Table 1. Features of *calcineurin B-likes* (*CBLs*) and *CBL-interacting kinases* (*CIPKs*) identified in *Solanum commersonii, S. tuberosum*, and *S. chacoense.* Strand and protein lengths (the number of amino acids) are reported. N/A - means not assigned.

Gene/protein	*S. commersonii*		*S. tuberosum*		*S. chacoense*	
	strand	length	strand	length	strand	length
CBL1-like	-	214	-	213	-	223
CBL2-like	+	223	-	247	+	220
CBL3-like	+	225	+	262	-	225
CBL4a	-	215	+	200	+	215
CBL4b	-	215	-	426	+	215
CBL5a	-	120	+	109	+	120
CBL5b		99	N/A	N/A	N/A	N/A
CBL8a	N/A	N/A	-	159	-	221
CBL8b	+	196	-	320	+	196
CBL9-like	+	262	-	215	-	214
CBL10	+	291	+	294	-	258
CIPK						
CIPK1-like	+	457	+	279	+	457
CIPK2-like	-	447	-	474	-	479
CIPK3a	+	439	+	546	+	438
CIPK3b	N/A	N/A	+	439	-	475
CIPK4-like	+	438	+	437	+	438
CIPK5-like	-	451	+	450	+	451
CIPK5b	N/A	N/A	N/A	N/A	+	451
CIPK6a	-	433	+	305	-	433
CIPK6b	-	425	+	424	+	402
CIPK7-like	-	434	-	433	-	434
CIPK8	-	448	+	468	+	448
CIPK9	-	409	-	406	-	452
CIPK11a	-	420	-	419	-	420
CIPK11b	+	447	-	1006	+	446
CIPK14-like	-	451	+	450	-	451
CIPK16-like	+	488	+	487	-	488
CIPK17-like	-	532	+	425	+	471
CIPK20a	-	459	-	461	-	463
CIPK20b	-	371	-	286	-	221
CIPK22-like	-	437	-	436	+	437
CIPK23	-	456	-	444	-	456
CIPK24	+	447	+	564	+	447
CIPK25	-	457	-	451	-	457
CIPK_specific1	-	471	+	447	-	471
CIPK_specific2	+	444	+	443	+	444
CIPK_specific3	+	442	+	153	N/A	N/A
CIPK_specific4	-	446	N/A	N/A	N/A	N/A

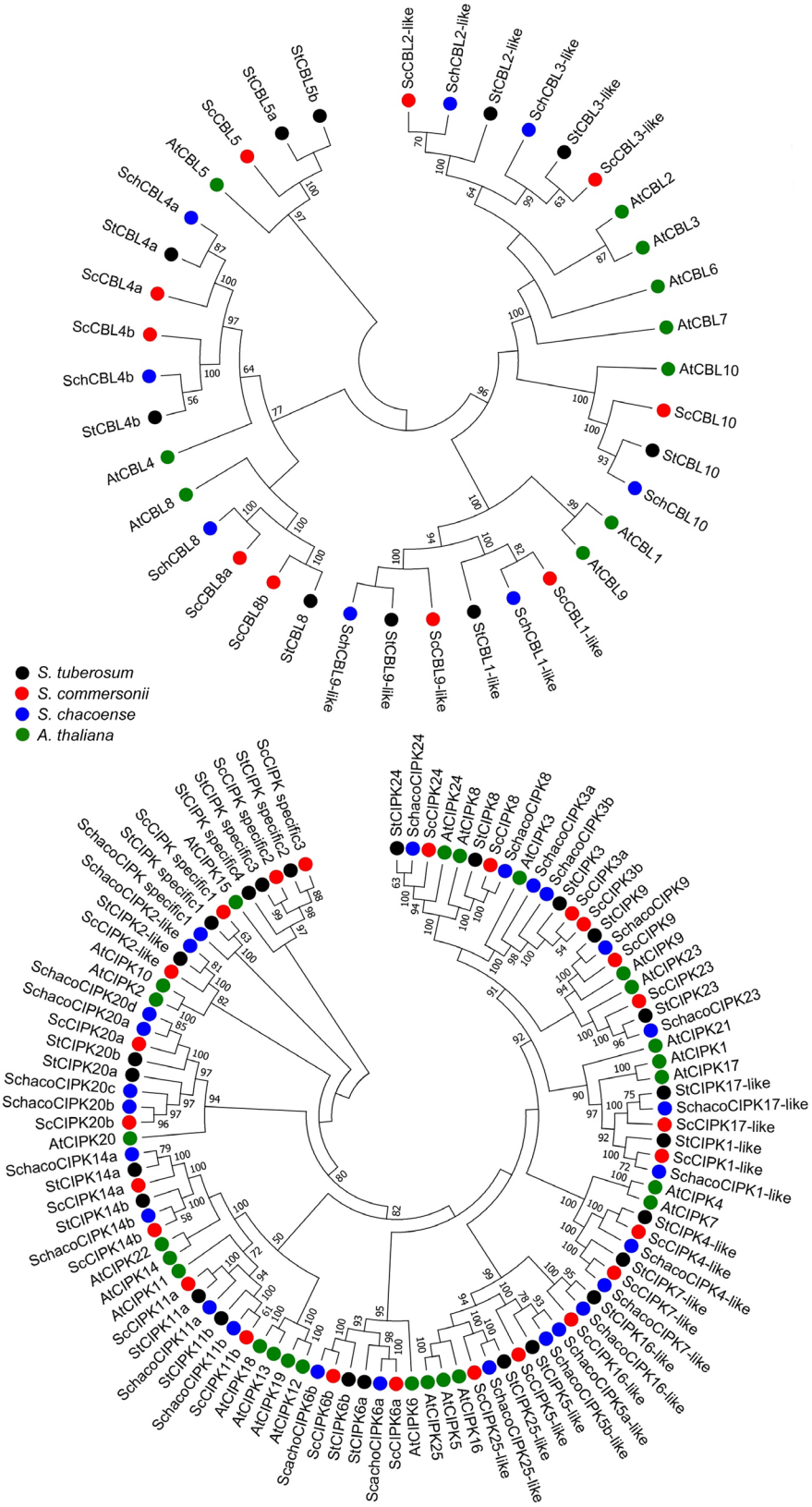

Fig. 1. Phylogenetic analysis of *calcineurin B-like* (*CBL*, *upper*) and *CBL-interacting kinase* (*CIPK*, *bottom*) gene families in *Solanum commersonii* (*red*), *S. chacoense* (*blue*), *S. tuberosum* (*black*), and *Arabidopsis thaliana* (*green*). Full length protein sequences of CBLs and CIPKs were used to construct maximum likewood trees by using the *MEGA7.0* software with a bootstrap value of 100 (only values > 50 are shown).

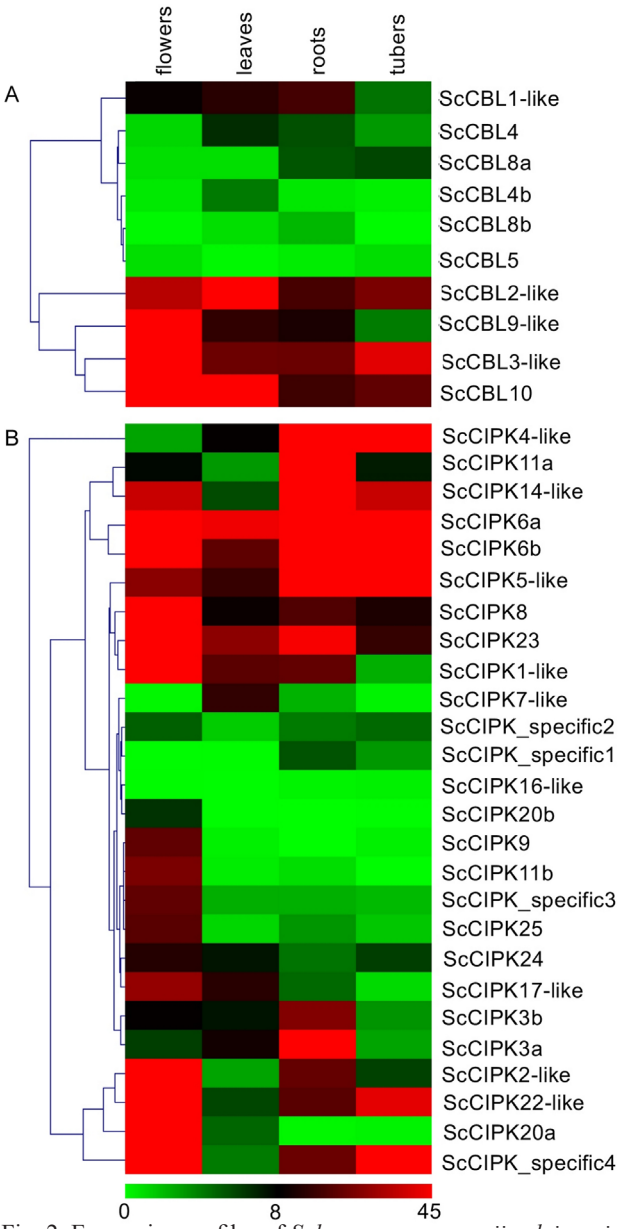

Fig. 2. Expression profiles of *Solanum commersonii calcineurin B-like* (*ScCBL*, *A*) and *S. commersonii CBL-interacting kinase* (*ScCIPK*, *B*) genes in four tissues (flowers, leaves, roots, and tubers). Data are reported as reads per kilobase of transcript per million mapped reads.

might function in membrane targeting of the CBLs.

Expression patterns of *ScCBLs* and *ScCIPKs* based on RNAseq data from four *S. commersonii* tissues (flowers, leaves, roots, and tubers) are reported as a heatmap in Fig. 2. The *ScCBL1-like*, *ScCBL2-like*, *ScCBL3-like*, *ScCBL9-like*, and *ScCBL10* were highly expressed throughout the various tissues in contrast to the others, which exhibited low expressions. In particular, *ScCBL3-like*, *ScCBL9-like*, and *ScCBL10* showed the highest expression in flowers whereas *ScCBL2-like* was highly expressed in leaves and *ScCBL4-like* in roots.

As far as *ScCIPKs* is concerned, four different groups were identified based on their expression patterns (Fig. 4*B*). The first group comprised seven *ScCIPKs* (*ScCIPK1-like*, *ScCIPK5-like*, *ScCIPK6a*, *ScCIPK6b*, *ScCIPK8*, *ScCIPK14-like*, and *ScCIPK23*), which were highly expressed in all tissues analyzed, whereas the second group included *ScCIPKs* with a low abundancy (*ScCIPK7-like*, *ScCIPK_specific1*, *ScCIPK_specific2*, *ScCIPK16-like*, and *ScCIPK20b*). The third group included five *ScCIPKs* (*ScCIPK9*, *ScCIPK11b*, *ScCIPK20b*, *ScCIPK25*, and *ScCIPK_specific3*), which showed expressions in flowers but not in the other tissues. The fourth group of *ScCIPKs* contained those highly expressed in all tissues excepted in leaves (*ScCIPK2-like*, *ScCIPK20a*, *ScCIPK22-like*, and *ScCIPK_specific4*). Only *ScCIPK4-like* and *ScCIPK11a* showed a high expression in roots but not in the other tissues. Collectively, these data indicate that *S. commersonii CBLs* and *CIPKs* might participate in specific functions in different organs.

Subsequently, we wondered whether *CBLs* and *CIPKs* play roles under different stresses. To date expression data on *S. commersonii* tissues exposed to any stress are not available. Since orthologous genes are known to retain equivalent functions in different organisms, we retrieved *CBLs* and *CIPKs* RPKM values from *S. tuberosum* experiments involving several stresses. As shown in Fig. 3, transcriptions of many *CBL* and *CIPK* genes altered in response to the various treatments, by contrast, only *CBL5*, *CIPK9*, and *CIPK16* did not change their transcriptional activity following stress conditions. *CBL1-like*, *CBL3-like*, *CBL9-like*, *CBL10*, and *CIPK17* increased their expression following heat treatment whereas the expressions of *CBL10*, *CIPK2*, *CIPK5*, *CIPK20a*, *CIPK11a* were altered in both salt conditions and mannitol conditions. The *CBL2-like*, *CBL10*, *CIPK1-like*, *CIPK3*, *CIPK4-like*, *CIPK6a*, *CIPK6b*, *CIPK8*, *CIPK14-like*, and *CIPK24* responded to 6-benzylaminopurine treatment whereas the other hormone treatments did not affect the expression of either *CBLs* or *CIPKs* (Fig. 3).

Further analysis will be carried out to assess the role of *CBL* and *CIPK* genes in *S. commersonii* challenged to abiotic and biotic stress conditions. The CIPKs have been demonstrated to interact with CBLs and several transporters to mediate multiple biological processes (Yang *et al.* 2008, Held *et al.* 2011, Huertas *et al.* 2012). Therefore, to further understand the interactions between ScCBL and ScCIPK proteins, an interaction network was built using the on-line tool *STRING* (Fig. 4). The homologous proteins with the highest bit score were considered as *STRING* queries. Using this approach, a total of 184 different interactions were found with an average of 13.6 for each protein pairs. The putative CIPK partners include CBLs involved in calcium ion binding, *Arabidopsis* K^+ transporter (AKT), salt overly sensitive 1 (SOS1) related to sodium-hydrogen transport, abscisic acid-insensitive 1-2 (ABI1 and ABI2) involved in protein dephosphorylation, calcium exchanger 1 (CAX1) related to calcium ion transport, and other proteins with binding activity. Five different CIPKs (CIPK1-like, CIPK3, CIPK7-like, CIPK9, and CIPK23) were found to putatively interact widespread with CBL1-like, CBL2-like, CBL3-like, CBL9, and CBL10 whereas CIPK5-like

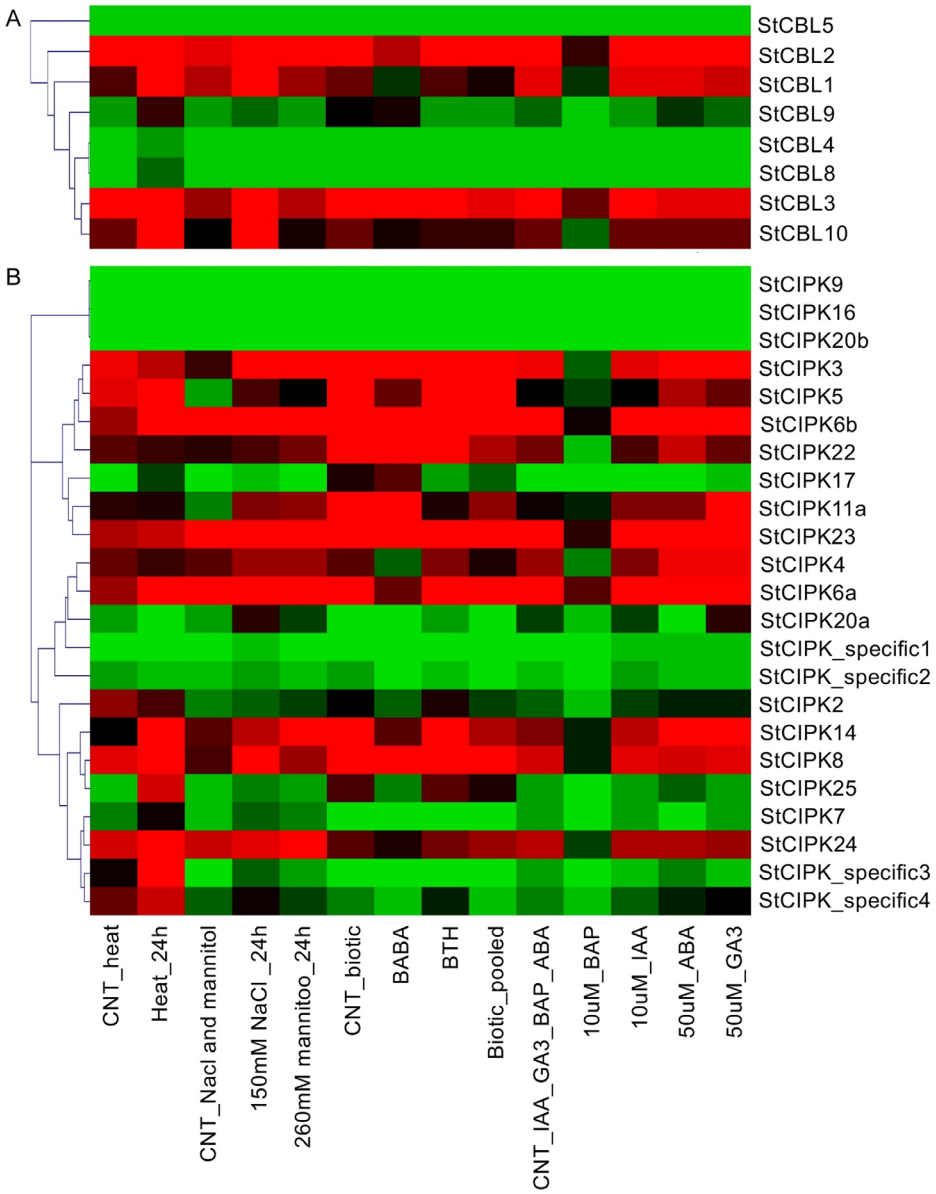

Fig. 3. Expression profiles of *Solanum commersonii calcineurin B-like* (*StCBL*) (*A*) and *S. commersonii CBL-interacting kinase* (*StCIPK*) (*B*) genes in response to heat, salt, mannitol, β-aminobutyric acid (BABA), benzothiadiazole (BTH), 6-benzylaminopurine (BAP), control (CNT), indole-3-acetic acid (IAA), abscisic acid (ABA), and gibberellic acid (GA_3) tretments.

and CIPK14-like are predicted to interact specifically with CBL2-like and CBL9and CIPK20 interact only with CBL9. Previous functional analyses have clarified that these interactions may participate in the regulation of plant ions transport, developmental processes, hormone signaling, and abiotic stress responses (Zhang *et al.* 2014). However, further molecular analyses are needed to clarify these interactions in *S. commersonii*.

Discussion

Calcium ions play a crucial role as second messengers in mediating various adaptive responses in plants under environmental stresses (Tuteja *et al.* 2007, Edel *et al.* 2017). Transient Ca^{2+} changes are sensed by several Ca^{2+}-binding proteins. Among them, the complex CBL-CIPK is the most interesting in transmitting stress signals from outside of the cell cytosol and to the nucleus. Despite extensive studies are available on the CBL-CIPK network in plant models, such as *Arabidopsis*, rice, and maize (Kolukisaoglu *et al.* 2004, Zhang *et al.* 2016), there are no reports regarding a comprehensive analysis of these two gene families in potato.

In the present study, 10 *CBL* genes were identified in the genome of the cultivated potato *S. tuberosum* and in its wild relatives *S. commersonii* and *S. chacoense*. A previous study by Mohanta *et al.* (2015) already reported the presence of many *CBLs* in *S. tuberosum.* However, details and clues regarding their structure and evolution

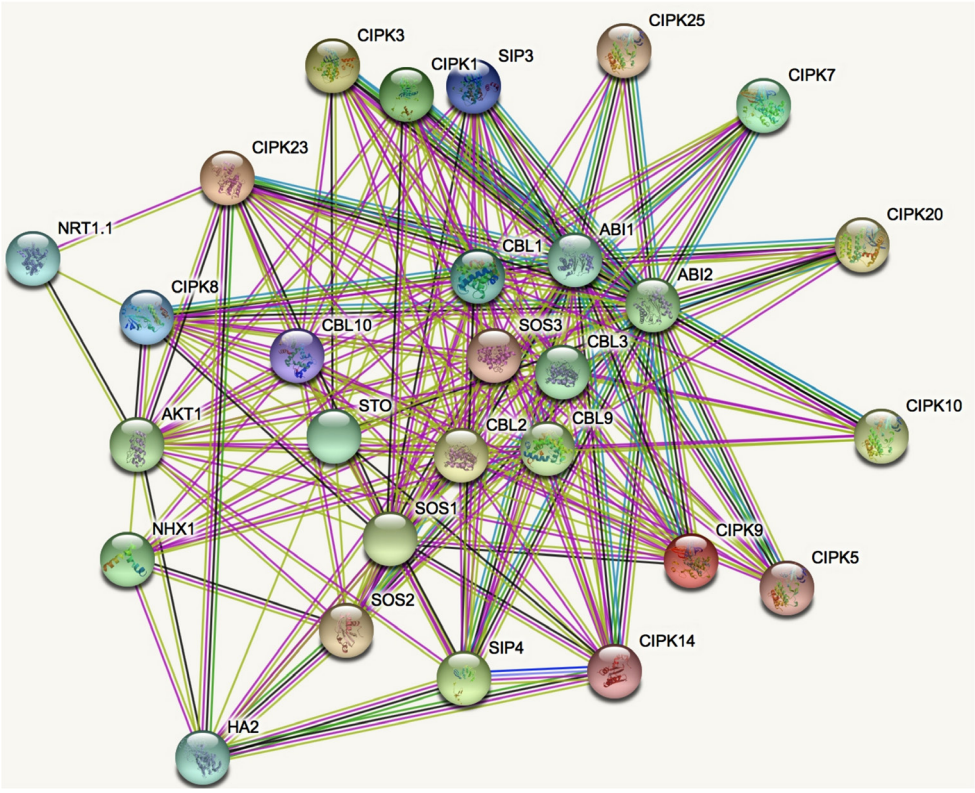

Fig. 4. A predicted interaction network of calcineurin B-like (CBLs) and CBL-interacting kinases (CIPKs) in *Solanum commersonii*. The CBL4 and CIPK24 are reported as salt overly sensitive (SOS)3 and SOS2, respectively.

are lacking. Ten *CBLs* were also identified in *Arabidopsis*, rice, and poplar (Kolukisaoglu *et al.* 2004, Yu *et al.* 2007). To date, the highest number of *CBLs* (13) has been identified in *Gossypium raimondii* (Mohanta *et al.* 2015) whereas the smallest (6) in *Sorghum bicolor* (Weinl *et al.* 2009). Our results on *CIPKs* confirm that this gene family in plants is larger compared to *CBL*. Indeed, we identified 26 *CIPKs* in the three potato genomes analyzed, 20, 26, and 30 *CIPKs* were found in *Vitis*, *Arabidopsis,* and rice, respectively (Kolukisaoglu *et al.* 2004, Kanvar *et al.* 2014, Xi *et al.* 2017). These findings indicate that gene duplication events, such as segmental and/or tandem duplications might play a significant role in their expansion. For such reasons, once genes were identified, we analyzed the relative contributions of both tandem duplications and segmental duplications. These mechanisms represent the two major forces driving gene evolution (Kong *et al.* 2007). Our results indicate that both *CBL* and *CIPK* genes originate from tandem duplication events. In contrast, Xi *et al.* (2017) reported that 50 % of *VvCBLs* and 40 % of *VvCIPKs* arise by tandem duplication, and 25 % of *VvCIPKs* are derived from segmental duplications in *V. vinifera*. Our phylogenetic analysis provided evidence that variability in the number of paralogs within different potato species were present. For example, *CIPK3* had two paralogs in *S. commersonii* and *S. chacoense* but only one in *S. tuberosum* hinting that the cultivated potato might have lost a copy due to domestication events, as already reported Lam *et al.* (2010) in *G. max*. By contrast, two paralogs of *CIPK5* were identified in *S. chacoense* but not in the other species, underlining the idea that, in different plant species, the number of copies might expand through different mechanisms. As far as structural features are concerned, such as conserved motifs and binding domains in *S. commersonii*, our results are in line with those reported in *Arabidopsis* and other species (Kolukisaoglu *et al.* 2004, Lyzenga *et al.* 2013, Yu *et al.* 2014, Mohanta *et al.* 2015). For example, the recently characterized motif PFPF, which is important for the phosphorylation cascades of CBLs (Ames *et al.* 1997, Kanwar *et al.* 2014), was identified in all ScCBL protein sequences. In addition, searching other possible motifs that could be functionally important in fulfilling CBL roles, we found that 4 out of 10 ScCBLs harbor a conserved N-myristoylation motif (MGXXXS/T), whereas 8 out of 10 bear a typical palmitoylation site at the N-terminal. A recent paper by Saito *et al.* (2018) revealed that these modifications promote a stable membrane association of soluble proteins including CBLs. For example, plasma membrane targeting CBL1 and CBL9 in *Arabidopsis* requires a dual lipid modification by N-myristoylation and S-acylation (Batistic *et al.* 2008). N-myristoylation and S-acylation of CBL4 are also essential for regulation of the Na^+/H^+ antiporter salt overly sensitive 1 (SOS1) and the potassium channel *Arabidopsis* K^+ transporter 2 (AKT2) (Ishitani *et al.* 2000, Qiu *et al.* 2002, Held *et al.* 2011). These findings confirm an important role of dual lipid modifications of regulatory soluble proteins for the modulation of intrinsic

membrane protein activity. However, the requirement of dual lipid modifications for Ca^{2+}-regulated processes in general remains to be elucidated, and how widespread this targeting mechanism is in plant signaling processes remains to be determined.

The expression profile of a gene in a specific plant tissue provides clues related to its function under a particular conditions or a stage of growth and development. Using tissue-specific transcriptome analysis, Lu *et al.* (2018) identified key genes with different expressions in individual organs involved in *de novo* fatty acid biosynthesis that affect lipid metabolism and oil content in *Brassica napa* seeds. In the case of *CBLs*, it has been shown through promoter-β-glucuronidase activity that *CBL1* and *CBL9* are expressed in leaves, roots, flowers, and vascular tissues in *Arabidopsis* (Pandey *et al.* 2004, Cheong *et al.* 2007) whereas *CBL4* is mainly expressed in root tissue (Quan *et al.* 2007) and *CBL10* in the shoot tissue (Cheong *et al.* 2010). In our study, *ScCBL1-like* and *ScCBL9-like* confirmed high expressions throughout various tissues suggesting that these genes might have a similar function in different plant tissues. By contrast, *ScCBL10* was mainly expressed in flowers and *ScCBL4* in roots, indicating diverse functions for their products in plant growth and development. Our data are also in line with those showed by Shi *et al.* (2000) in *Arabidopsis*. In the case of *CIPKs,* we found that many *ScCIPKs* (*i.e.*, *ScCIPK1-like*, *ScCIPK5-like*, *ScCIPK6a*, *ScCIPK6b*, *ScCIPK8*, *ScCIPK14-like*, and *ScCIPK23*) were constitutively expressed in all tissues analyzed indicating that they may play widespread roles in these organs as already shown for other species such as *Triticum aestivum* and *Manihot esculenta* (Sun *et al.* 2015, Mo *et al.* 2018). By contrast, other *CIPK* genes were abundantly expressed in certain tissues. For example, *ScCIPK4* was mainly expressed in roots, as also reported Linn *et al.* (2017) in *Arabidopsis* whereas the expression of Sc*CIPK25* was detected in flowers and roots, consistent with data by Meena *et al.* (2018).

Previous studies demonstrated that the CBL-CIPK complex is involved in regulating response to environmental stresses (Kanwar *et al.* 2014, Yu *et al.* 2014). For example, *AtCBL10* was reported to interact with *AtCIPK24* in response to salt stress (Kim *et al.* 2007) and was shown to negatively compete with *AtCIPK23* for binding to *Arabidopsis* K^+ transporter (AKT1), modulating its activity (Huertas *et al.* 2012, Ren *et al.* 2013). Our dataset analysis revealed that *ScCBL10* (orthologous of *AtCBL10*) together with *ScCBL3* and *ScCBL9* increased its expression following heat stress. The *CBL3* has been reported to be induced also in rice upon heat stress (Mohanta *et al.* 2015) whereas *CBL9* was activated in *Brassica napus* (Zhang *et al.* 2014) following the same stress conditions. These results reinforce the hypothesis that more than one *CBL* might participate in regulating the same stress conditions. Other stress responsive *CBLs* are *CBL5* and *CBL4.* Indeed, in 2010, Cheong *et al*. (2010) provided evidence that *AtCBL5* confers drought tolerance whereas Qiu *et al.* (2002) showed that *AtCBL4* is involved in salt tolerance. In contrast to these results, ortholog genes *ScCBL4* and *ScCBL5* did not change their expressions under all the stress conditions studied here. These data support that CBL/CIPK signaling components may have different functions in responding stress conditions in different plant species.

Conclusions

In the present study, we reported for the first time a genome wide analysis of *CBLs* and *CIPKs* in *S. tuberosum*, *S. commersonii*, and *S. chacoense.* The identification of 10 *CBLs* and 26 *CIPKs* suggests that the complexity of the CBL-CIPK signaling network in potato is similar to that reported in other species. The analysis of evolutionary features of these genes revealed that tandem duplication events were the main evolutionary forces underlying the expansion of both gene families. Finally, the investigation of the role of these genes upon stress conditions pointed out that *S. commersonii CBLs* and *CIPKs* exhibited differential responses to multiple stimuli. All these findings increase our understanding on the calcium signal pathways in potatoes, paving the way for future breeding approaches.

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