WRKY genes family study reveals tissue-specific and stress-responsive TFs in wild potato species

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Wild potatoes, as dynamic resource adapted to various environmental conditions, represent a powerful and informative reservoir of genes useful for breeding efforts. WRKY transcription factors (TFs) are encoded by one of the largest families in plants and are involved in several biological processes such as growth and development, signal transduction, and plant defence against stress. In this study, 79 and 84 genes encoding putative WRKY TFs have been identified in two wild potato relatives, *Solanum commersonii* and *S. chacoense*. Phylogenetic analysis of WRKY proteins divided ScWRKys and SchWRKys into three Groups and seven subGroups. Structural and phylogenetic comparative analyses suggested an interspecific variability of WRKYs. Analysis of gene expression profiles in different tissues and under various stresses allowed to select ScWRKY045 as a good candidate in wounding-response, ScWRKY055 as a bacterial infection triggered WRKY and ScWRKY023 as a multiple stress-responsive WRKY gene. Those WRKys were further studied through interactome analysis allowing the identification of potential co-expression relationships between ScWRKys/SchWRKys and genes of various pathways. Overall, this study enabled the discrimination of WRKY genes that could be considered as potential candidates in both breeding programs and functional studies.

Plants experience environmental constrains and pathogen attacks during their life. Being sessile organisms, their survival depends on the ability to properly and promptly reprogram cellular networks. Several and different classes of transcription factors (TFs) work as “master regulators” and “selector genes”, being able to control processes that specify cell types and developmental patterning and modulate specific pathways. Among them, WRKY factors are drawing a great deal of interest in the scientific community due to their ability to simultaneously cope with multiple stresses1,2. They are notorious for coordinating signals in plant immunity response against several pathogens and pest attacks3,4. More recently, it has been confirmed that WRKYS also base defence mechanism to abiotic stresses and play a key role in cross-talk pathway networks between plant response and development5,6. Their involvement into multiple stress response and in plant growth regulation is evidenced by their W-box specific DNA binding7,8. Besides, WRKY binds sugar responsive elements and, very recently, it has been demonstrated that they activate sugar responsive genes through an epigenetic mechanism of control9. The systematic classification of components of the WRKY family is well organized. It is based on the WRKY binding domain (WD) characteristics along with those of the Zinc Finger (ZF) motif, which is typically present downstream the WD. WD consists of 60 amino acids structured as four-stranded 3-sheets able to enter the major groove of B-form DNA. The highly conserved motif is "WRKYGQK". According to the number of WDs and the type of zinc finger motif, WRKY proteins can be classified into three Groups, namely Group I, II, and III: Group I WRKY members contain two WDs with two classical C2H2 ZF motifs, Group II WRKYs have one WD with one C2H2 ZF motif, and Group III WRKYS contain one WD with one C2HC ZF motif3,5. Group II WRKYS can be divided into five subGroups (Ia-Ie)10. It is well recognized that Group I WRKY members are the evolutionary ancestors of the other WRKYS and that they exist only in lower plants11,12. The complexity of this gene family involves different molecular levels, from the transcriptional self-regulation through microRNAs to post-transcriptional events, such as alternative splicing, post-translational regulation through ubiquitin proteasome system and MAPK cascade3. Studies addressed to mine sequence divergences or to identify gene expression differences in WRKYS of...
cultivated and wild species are increasing. Such investigations may pave the way into exploiting these regulators for breeding purposes. A recent study carried out in the sweet potato wild ancestor *Ipomoea trifida*, highlighted how investigations on WRKY gene family in wild relatives can boost the molecular breeding of cultivated species\(^2\). However, our knowledge is still not complete and therefore WRKY gene biodiversity remains unlocked in many species.

The potato, *Solanum tuberosum*, is one of the most cultivated non-cereal crop in the world. Its cultivation is often hampered by the fact that it is susceptible to a wide range of stressors causing severe yield losses. Sources of resistance can be found in its tuber-bearing wild relatives, that are highly used as rootstock for cultivated Solanaceae\(^14\) but poorly used in breeding programs. However, recent technologies can be implemented to enhance resistance can be found in its tuber-bearing wild relatives, that are highly used as rootstock for cultivated Solanaceae\(^14\) but poorly used in breeding programs. However, recent technologies can be implemented to enhance resistance.

The exon-intron organization of WRKY genes was determined using the online GSDS tool (http://gsds.cbi.pku.edu.cn). Finally, the online tool26 was first used to establish the best-fit model of evolution through the option “Find best DNA/Protein Models” implemented in the program and then for phylogenetic tree building using the appropriate options. In the phylogenetic analysis were integrated seven AtWRKY proteins randomly selected as representative of each WRKY Group, as already reported by Karanja et al.\(^28\). One-to-one orthologs were considered when candidate proteins allocated on the same clade in the phylogenetic tree with *S. tuberosum*. The exon-intron organization of WRKY genes was determined using the online GSDS tool (http://gsds.cbi.pku.edu.cn). Finally, the on-line tool Phenogram (http://visualization.ritchielab.org/phenograms/plot) was used to determine the location of the WRKY genes on *S. chacoense* chromosomes.

**Materials and Methods** Identification of WRKY in *S. commersonii* and *S. chacoense* and phylogenetic analysis. The well-known WRKY protein sequences of *S. tuberosum*\(^28\) and *A. thaliana*\(^29\) were used as queries to build an HMM profile through HMMER as reported by Esposito et al.\(^28\) and to search orthologs in *S. commersonii* (cmmt1T clone of PI243503) and *S. chacoense* (M6 clone) genomes. Only sequences with an e-value lower than 10\(^{-5}\) and an identity higher than 55% were regarded as putative WRKYs and further analyzed. The full-length WRKY candidate proteins were then manually confirmed by checking the WRKY domain using the NCBI search domain online tool\(^26\) and used for the phylogenetic analysis. Names were assigned based on *S. tuberosum* orthologs using bootstrap replicates of the Maximum Likelihood (ML) phylogenetic tree (values higher than 50). Briefly, MEGAX\(^26\) was first used to establish the best-fit model of evolution through the option “Find best DNA/Protein Models” implemented in the program and then for phylogenetic tree building using the appropriate options. In the phylogenetic analysis were integrated seven AtWRKY proteins randomly selected as representative of each WRKY Group, as already reported by Karanja et al.\(^28\).

**Public RNaseq-based expression analysis.** The transcriptional activity of WRKY genes related to three tissues (flower, leaf and tuber) in *S. commersonii* and *S. chacoense* was estimated using the publicly available RNAseq data sets. As far as *S. commersonii* is concerned, we used raw single-end fastq data deposited under study SRP050412. Briefly, to remove unwanted sequences originating from organelles, reads were mapped against the mitochondrial (*S. tuberosum* Group_Phereja mitochondrial_DM1-3-516-R44) and chloroplast (*S. tuberosum* Group_Phereja chloroplast_DM1-3-516-R44) genomes using BOWTIE2\(^22\) with sensitive local mapping. Unmapped reads were mapped against the *S. commersonii* genome. The BAM files were then analyzed using Cufflinks–Cuffquant software (version 2.2.1) to assemble the aligned reads and to access transcriptome complexity. Expression values for each gene were estimated based on RPKM (Reads Per Kilobase of transcript per Million mapped reads) using the default options. No biological replicates were available for *S. commersonii*. As for *S. chacoense*, data were expressed as mean of biological replicates and RPKM values we directly retrieved from SpudDB (http://solanaceae.plantbiology.msu.edu). For all StWRKY orthologs we recovered from the public *S. tuberosum* database (http://solanaceae.plantbiology.msu.edu) transcriptional data regarding potato leaves subject to salt stress (50 mM NaCl for 24 h), osmotic stress (260 µM mannitol for 24 h), heat stress (35 °C for 24 h) and treatments with 6-benzylaminopurine (BAP) (10 µM for 24 h), abscisic acid (ABA) (50 µM for 24 h), indole-3-acetic acid (IAA) (10 µM for 24 h), gibberellic acid (GA3) (50 µM for 24 h), 3-aminobutyric acid (BABA) (24, 48, 72 h), benzothiadiazole (BTH) (24, 48, 72 h), and *in vitro* culture (root and shoot).

**Plant materials and stress treatments.** *In-vitro* plantlets of *S. commersonii* clone cmmt1T, accession PI243503, derived from the Inter-Regional Potato Introduction Station (Sturgeon Bay, Wisconsin), were micro-propagated as described by D’Amelio et al.\(^30\). Four-week-old vitroplants were transplanted into 14-mm plastic pots containing sterile soil and grown in a greenhouse under long-day conditions (16-h light, 8-h dark); temperature was set at 26 °C during the day and 18 °C at night. Three-week-old seedlings were used for all stress experiments and sampled in a 0, 1, 2, 4, 6 hpt (hours post treatment) time course. As for virus infection, young plants of clone cmmt1T were mechanically inoculated with Potato Virus Y tuber necrotic strain (PVY\(_{NTN}\)) as
reported by Esposito et al. For assessing bacterial resistance, the protocol of Melito et al. was used with few modifications. The stem base of vitroplants (one injection per plant) was inoculated with 20 µl of P. carotovorum strain Ecc 009 suspension under greenhouse conditions (with temperatures ranging from 20 to 30 °C during the day and from 12 to 17 °C during the night). The bacterial culture was adjusted to 10^6 CFU·mL^-1 in MgCl₂ solution. The whole plant was then covered with a transparent plastic bag. For both treatments (viral and bacterial), plants inoculated with buffer were considered as mock control. At each time point, leaves were collected from three biological replicates, both for treated and untreated samples. Each biological replicate consisted of a pool of three plants. Young leaf samples were collected from treated and mock control plants following the time course and stored at −80 °C before RNA extraction. Wounding stress was induced according to the protocol of Vannozzi et al. with few modifications. Leaf discs (15 mm diameter) were punched from healthy leaves detached from glasshouse-grown plantlets and incubated upside down on 3MM moist filter paper in large Petri dishes at 22 °C under 12h light / 12h dark conditions until harvest. Collected discs were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. Five discs were randomly chosen per each time point. No treated leaves were used as control. Each treatment consisted of three biological replications.

RNA extraction, cDNA synthesis and quantitative Real-Time PCR (RT-qPCR). Total RNA was isolated from 100 mg of grinded leaves as reported by Rinaldi et al. and Villano et al. The Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) was used following the manufacturer’s protocol with some modifications. Quantity and quality of the isolated RNA was measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). cDNA synthesis, 1 µg of each RNA sample was reverse transcribed using the SuperScript III cDNA Synthesis Kit (Life Technologies, Paisley, UK) following the manufacturer’s protocol. Specific primers were designed using the website Primer3 as reported by Koressaar et al. (Supplementary Table 1). Expression analysis was conducted by RT-qPCR as reported by Di Meo et al. and Brulè et al. using a SYBR Green method on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each 15 µl PCR reaction contained 330 nM of each primer, 2 µl of 5-fold diluted cDNA and 7.5 µl of SYBR Green Mix (Applied Biosystems, Foster City, CA, USA). The SDS 2.3 and RQ Manager 1.2 software (both Applied Biosystems, Foster City, CA, USA) were used for data elaboration. The expression of each target gene was normalized with the expression level of the housekeeping gene (Elongation Factor) and calibrated with the mock control using the Livak method, obtaining the values in log₂(FC). Each analysis consisted of three technical replications.

Protein-protein interaction in silico analyses. An interactome analysis was carried out to investigate the function of tissue-specific and stress responsive ScWRKYs and SchWRKYs selected in the expression study through the analysis of direct ortholog of StWRKY genes. The protein-protein interaction networks STRING database was used (http://string-db.org/). It reports protein associations based on various sources, such as experimental results, pathway understanding, text-mining and genomic information. The interactome was constructed using a medium confidence score (0.400).

Results

Phylogenetic analysis and classification of ScWRKYs and SchWRKYs. A total of 79 and 84 candidates corresponding to the Pfam WRKY family were distinguished in S. commersonii and S. chacoense, respectively (Table 1). Based on phylogenetic analysis, 71 ScWRKYs and 80 in SchWRKYs were identified as direct orthologs of StWRKYs, while the remaining were classified as not direct orthologs and named with the suffix -a and -b (Table 1). The phylogenetic analysis of seven AtWRKY proteins randomly selected as representative of each WRKY Group and all S. commersonii and S. chacoense WRKY proteins revealed ScWRKY and SchWRKY classification in three large Groups corresponding to Group I, II and III (Fig. 1), with the exception of nine proteins in S. commersonii (ScWRKY047, ScWRKY051, ScWRKY052, ScWRKY055, ScWRKY085, ScWRKY087a, ScWRKY087b, ScWRKY088 and ScWRKY089) and eight proteins in S. chacoense (SchWRKY047, SchWRKY051, SchWRKY052, SchWRKY056, SchWRKY057, SchWRKY085, SchWRKY088 and SchWRKY089), that were not assigned to any Group (Table 1). In S. commersonii, 12 ScWRKY proteins belonged to Group I, 47 to Group II, and 10 to Group III. Group II proteins were further categorized into subGroups. Group Ia, Ib, Ic, Id and Ie included 5, 8, 13, 7 and 14 ScWRKYs respectively (Table 1). As far as S. chacoense is concerned, 14 proteins belonging to Group I, 45 to Group II, and 15 to Group III were identified. Those of Group II were classified in subGroup Iia (5 ScWRKYs), Iib (5), Iic (15), Id (7) and Ie (12) (Table 1). Gene and protein features, including the length of the protein sequence, the WRKY domain motif composition and the exons/introns number were analyzed and reported in Supplementary Table 2. In S. commersonii, the “WRKYGQK” pattern was highly conserved in 69 ScWRKYs, while five variations were observed in the other proteins (“WGKYQGK”; “WRWLKCG”; “WSKYGMK”; “WKGYGQK”; “WKGYQGK”). In S. chacoense, 74 ScWRKYs contained the “WRKYGQK” domain, while the other proteins contained one of the following variations: “WIKYGQK”, “WHKYGQK”, “WKCGYQK”, “WKHGSN”, “WHKCGQK”.” Concerning the Zinc Finger motif, the most common pattern in both species was “C-X₄₋₅₋₇₋C-X₁₂₋₂₃₋₂₄₋H₋X-H/C”. The only exceptions were ScWRKY068 with “C-X₃₋C-X₁₋H-X₁₋H/C”, ScWRKY074 with “C-X₁₋C-X₁₂₋H-X-C”, and ScWRKY074 with “C-X₃₋C-X₁₋H-X-C”. Regarding the number of WDs in the studied proteins, out of 12 members belonging to Group I in S. commersonii, eight contained two WDs and two other two possessed three WDs. All Group II members in S. chacoense harbored two WDs, except SchWRKY014 (one WD). Seven ScWRKYs belonging to Group II and two of Group III contained two WDs, while all other members had only one WD. In S. chacoense, Group II and III proteins harbored one WD. All Group III members contained the H₂C Zinc Finger domain (Supplementary Table 2). Our analysis pointed out that the number of amino acids of ScWRKYs varied from 107 (ScWRKY30) to...
| S. tuberosum WRKYs | S. commersonii WRKYs | Locus ID ScWRKYs | ScWRKY Groups | S. chacoense WRKYs | Locus ID SchWRKYs | SchWRKY Groups |
|------------------|---------------------|-----------------|---------------|------------------|-----------------|---------------|
| S. tuberosum WRKYs | S. commersonii WRKYs | Locus ID ScWRKYs | ScWRKY Groups | S. chacoense WRKYs | Locus ID SchWRKYs | SchWRKY Groups |
| S. tuberosum WRKY001 | S. commersonii WRKY001 | maker_scaffold1882_snap_gene_0_38_mRNA_1 | I | SchWRKY001 | g1877.t1 | I |
| S. tuberosum WRKY002 | S. commersonii WRKY002 | maker_scaffold7854_augustus_gene_0_54_mRNA_1 | I | SchWRKY002 | g1307.t1 | I |
| S. tuberosum WRKY003 | S. commersonii WRKY003 | maker_scaffold2503_augustus_gene_0_43_mRNA_1 | I | SchWRKY003 | g1632.t1 | I |
| S. tuberosum WRKY004 | - | - | - | - | - | - |
| S. tuberosum WRKY005 | S. commersonii WRKY005 | maker_scaffold31249_augustus_gene_0_94_mRNA_1 | I | SchWRKY005 | g9688.t1 | I |
| S. tuberosum WRKY006 | S. commersonii WRKY006 | augustus_masked_scaffold354_abinit_gene_0_10_mRNA_1 | I | SchWRKY006 | g2882.t1 | I |
| S. tuberosum WRKY007 | - | - | - | - | - | - |
| S. tuberosum WRKY008 | S. commersonii WRKY008 | maker_scaffold9215_augustus_gene_0_73_mRNA_1 | I | SchWRKY008 | g53550.t1 | I |
| S. tuberosum WRKY009 | - | - | - | - | - | - |
| S. tuberosum WRKY010 | S. commersonii WRKY010 | maker_scaffold440_augustus_gene_0_51_mRNA_1 | I | SchWRKY010 | g35137.t1 | I |
| S. tuberosum WRKY011 | S. commersonii WRKY011 | maker_scaffold1729_augustus_gene_0_61_mRNA_1 | I | SchWRKY011 | g18246.t1 | I |
| S. tuberosum WRKY012 | S. commersonii WRKY012 | generemark_scaffold41213_abinit_gene_0_8_mRNA_1 | I | SchWRKY012 | g1746.t1 | I |
| S. tuberosum WRKY013 | S. commersonii WRKY013 | generemark_scaffold21247_abinit_gene_0_14_mRNA_1 | I | SchWRKY013 | g2208.t1 | I |
| S. tuberosum WRKY014 | S. commersonii WRKY014 | augustus_masked_scaffold89_abinit_gene_0_4_mRNA_1 | I | SchWRKY014 | g31999.t1 | I |
| S. tuberosum WRKY015 | - | - | - | - | - | - |
| S. tuberosum WRKY016 | S. commersonii WRKY016 | maker_scaffold31159_snap_gene_0_73_mRNA_1 | IIb | SchWRKY016 | g32923.t1 | IIc |
| S. tuberosum WRKY017 | S. commersonii WRKY017 | maker_scaffold17033_augustus_gene_1_25_mRNA_1 | IIb | SchWRKY017 | g9538.t1 | IIa |
| S. tuberosum WRKY018 | S. commersonii WRKY018 | maker_scaffold1729_augustus_gene_0_61_mRNA_1 | IIb | SchWRKY018 | g53550.t1 | IIa |
| S. tuberosum WRKY019 | S. commersonii WRKY019 | maker_scaffold11314_augustus_gene_0_23_mRNA_1 | IIb | SchWRKY019 | g8360.t1 | IIa |
| S. tuberosum WRKY020 | - | - | - | - | - | - |
| S. tuberosum WRKY021 | S. commersonii WRKY021 | maker_scaffold31159_snap_gene_0_73_mRNA_1 | IIa | SchWRKY021 | g1533.t1 | III |
| S. tuberosum WRKY022 | S. commersonii WRKY022 | maker_scaffold2968_augustus_gene_0_60_mRNA_1 | IIa | SchWRKY022 | g16975.t1 | III |
| S. tuberosum WRKY023 | S. commersonii WRKY023 | maker_scaffold9305_augustus_gene_0_17_mRNA_1 | IIa | SchWRKY023 | g5351.t1 | III |
| S. tuberosum WRKY024 | S. commersonii WRKY024 | maker_scaffold27786_augustus_gene_0_4_mRNA_1 | IIa | SchWRKY024 | g31307.t1 | III |
| S. tuberosum WRKY025 | S. commersonii WRKY025 | maker_scaffold38372_augustus_gene_0_21_mRNA_1 | IIa | SchWRKY025 | g31307.t1 | III |
| S. tuberosum WRKY026 | - | - | - | - | - | - |
| S. tuberosum WRKY027 | S. commersonii WRKY027 | maker_scaffold13399_snap_gene_0_85_mRNA_1 | IIc | SchWRKY027 | g6153.t1 | III |
| S. tuberosum WRKY028 | S. commersonii WRKY028 | maker_scaffold1174_augustus_gene_0_6_mRNA_1 | IIc | SchWRKY028 | g2362.t1 | III |
| S. tuberosum WRKY029 | S. commersonii WRKY029 | maker_scaffold7139_snap_gene_0_53_mRNA_1 | IIc | SchWRKY029 | g14938.t1 | III |
| S. tuberosum WRKY030 | S. commersonii WRKY030 | maker_scaffold7139_snap_gene_0_53_mRNA_1 | IIc | SchWRKY030 | g14938.t1 | III |
| S. tuberosum WRKY031 | S. commersonii WRKY031 | maker_scaffold13399_snap_gene_0_85_mRNA_1 | IIc | SchWRKY031 | g592.t1 | III |
| S. tuberosum WRKY032 | S. commersonii WRKY032 | maker_scaffold45864_snap_gene_0_45_mRNA_1 | IIc | SchWRKY032 | g895.t1 | III |
| S. tuberosum WRKY033 | - | - | - | - | - | - |
| S. tuberosum WRKY034 | S. commersonii WRKY034 | generemark_scaffold11173_abinit_gene_0_31_mRNA_1 | IIc | SchWRKY034 | g10072.t1 | III |
| S. tuberosum WRKY035 | S. commersonii WRKY035 | maker_scaffold23185_snap_gene_0_11_mRNA_1 | IIc | SchWRKY035 | g2067.t1 | III |
| S. tuberosum WRKY036 | S. commersonii WRKY036 | maker_scaffold768_snap_gene_0_64_mRNA_1 | IIc | SchWRKY036 | g969.t1 | III |
| S. tuberosum WRKY037 | S. commersonii WRKY037 | maker_scaffold230_augustus_gene_0_11_mRNA_1 | IIc | SchWRKY037 | g1078.t1 | III |
| S. tuberosum WRKY038 | - | - | - | - | - | - |
| S. tuberosum WRKY039 | S. commersonii WRKY039 | maker_scaffold24623_snap_gene_0_85_mRNA_1 | IIc | SchWRKY039 | g2454.t1 | III |
| S. tuberosum WRKY040 | - | - | - | - | - | - |

Continued
The exon-intron organization of our WRKY genes was examined to gain more insight into the evolution of the WRKY family in potato. As shown in Supplementary Table 2, all ScWRKY genes possessed from one to eight exons. A similar trend was observed in S. chacoense. Concerning the genomic localization of WRKY genes, due to the unavailability of S. commersonii physical map, we plotted genes only on S. chacoense chromosomes using the Phenogram online tool (http://visualization.ritchielab.org/phenograms/plot) (Figure S1). Out of 84 SchWRKY genes identified, 83 were mapped. As represented in Figure S1, most of the genes were located on chromosome 3 (11 genes; 13.1%), followed by chromosome 5 (10; 11.9%), Unknown (8; 9.5%) and 2 (7; 8.3%). A total of 25 SchWRKY genes (5 on each chromosome) were localized on chromosomes 7 to 12, whereas no one was mapped on chromosome 11.

Expression patterns of WRKY genes in S. commersonii and S. chacoense. To explore the expression of WRKY genes, we analyzed and calculated the RNA sequence data available for leaf, flower and tuber in both species (Figs. 2a and 2b). The heat-map based expression profiles of ScWRKYs (Fig. 2a) and SchWRKYs (Fig. 2b) revealed their dynamic and differential expression in various tissues and that the range of expression

| S. tuberosum WRKYs | S. commersonii WRKYs | Locus ID ScWRKYs | ScWRKY Groups | S. chacoense WRKYs | Locus ID SchWRKYs | SchWRKY Groups |
|--------------------|----------------------|------------------|----------------|-------------------|-----------------|----------------|
| StWRKY054          | ScWRKY054            | maker_scaffold16944_snap_gene_0_17_mRNA_1 | IIIe | SchWRKY054       | g22375.t1       | IIId |
| StWRKY055          | ScWRKY055            | maker_scaffold15104_snap_gene_0_20_mRNA_1 | n.a. | SchWRKY056       | g9378.t1       | n.a. |
| StWRKY056          | -                    | -                | -              | SchWRKY057       | g9376.t1       | n.a. |
| StWRKY058          | ScWRKY058-like       | maker_scaffold19913_augustus_gene_0_9_mRNA_1 | IIId | -                | -              | -              |
| StWRKY059          | ScWRKY059            | maker_scaffold174_augustus_gene_0_58_mRNA_1 | IIIe | SchWRKY059       | g15764.t1       | IIIe |
| StWRKY060          | augustus_masked_scaffold18408_abinit_gene_0_2_mRNA_1 | IIle | SchWRKY060       | g30025.t1       | IIle |
| StWRKY061          | genemark_scaffold32401_abinit_gene_0_3_mRNA_1 | IIle | SchWRKY061       | g24860.t1       | IIle |
| StWRKY062          | augustus_masked_scaffold5103_abinit_gene_0_3_mRNA_1 | IIle | SchWRKY062       | g9225.t1       | IIle |
| -                  | ScWRKY062-like       | maker_scaffold1081_snap_gene_0_34_mRNA_1 | IIId | -                | -              | -              |
| StWRKY063          | ScWRKY063            | maker_scaffold1081_snap_gene_0_35_mRNA_1 | IIle | SchWRKY063       | g9218.t1       | IIle |
| StWRKY064          | ScWRKY064            | maker_scaffold17826_snap_gene_0_36_mRNA_1_like | IIle | SchWRKY064       | g32306.t1       | IIle |
| StWRKY065          | genemark_scaffold17826_abinit_gene_0_25_mRNA_1 | IIle | SchWRKY065       | g9511.t1       | IIle |
| StWRKY066          | ScWRKY066-like       | maker_scaffold35381_snap_gene_0_8_mRNA_1 | IIId | -                | -              | -              |
| StWRKY067          | ScWRKY067            | maker_scaffold1552_snap_gene_0_60_mRNA_1 | IIIe | SchWRKY067       | g9511.t1       | IIIe |
| StWRKY068          | ScWRKY068            | genemark_scaffold25887_abinit_gene_0_18_mRNA_1 | III | SchWRKY068       | g21153.t1       | III |
| StWRKY069          | -                    | -                | -              | SchWRKY069       | g24755.t1       | III |
| StWRKY070          | ScWRKY070            | maker_scaffold30616_augustus_gene_0_60_mRNA_1 | III | SchWRKY070       | g13833.t1       | III |
| StWRKY071          | ScWRKY071            | maker_scaffold12441_snap_gene_0_28_mRNA_1 | III | SchWRKY071       | g30258.t1       | III |
| StWRKY072          | ScWRKY072            | maker_scaffold12583_augustus_gene_0_32_mRNA_1 | III | SchWRKY072       | g4219.t1       | III |
| StWRKY073          | -                    | -                | -              | SchWRKY073       | g9466.t1       | III |
| StWRKY074          | ScWRKY074            | maker_scaffold31861_augustus_gene_0_50_mRNA_1 | III | SchWRKY074       | g9394.t1       | III |
| StWRKY075          | -                    | -                | -              | SchWRKY075       | g9693.t1       | III |
| StWRKY076          | ScWRKY076            | snap_masked_scaffold31861_abinit_gene_0_36_mRNA_1 | III | SchWRKY076       | g9390.t1       | III |
| StWRKY077          | -                    | -                | -              | SchWRKY077       | g5563.t1       | III |
| StWRKY078          | ScWRKY078            | maker_scaffold978_augustus_gene_1_25_mRNA_1 | III | SchWRKY078       | g22449.t1       | III |
| StWRKY079          | ScWRKY079            | maker_scaffold3600_augustus_gene_0_37_mRNA_1 | III | SchWRKY079       | g32303.t1       | III |
| StWRKY080          | ScWRKY080            | maker_scaffold15162_snap_gene_0_46_mRNA_1 | III | SchWRKY080a      | novel_model_169_57a3878 | III |
| -                  | -                    | -                | -              | SchWRKY080b      | temp_model_12.1.57a3880 | III |
| StWRKY081          | ScWRKY081            | maker_scaffold7208_snap_gene_0_38_mRNA_1 | III | SchWRKY081       | g5357.t1       | III |
| StWRKY082          | ScWRKY082            | augustus_masked_scaffold568_abinit_gene_0_2_mRNA_1 | IIIe | SchWRKY082       | g37078.t1       | IIIe |
| -                  | ScWRKY083            | augustus_masked_scaffold687_abinit_gene_0_0_mRNA_1 | IIIe | SchWRKY083       | g15040.t1       | IIIe |
| -                  | ScWRKY084            | augustus_masked_scaffold10960_abinit_gene_0_3_mRNA_1 | IIIa | -                | -              | -              |
| -                  | ScWRKY084-like       | maker_scaffold5413_augustus_gene_0_50_mRNA_1 | IIIb | -                | -              | -              |
| -                  | ScWRKY085            | augustus_masked_scaffold12000_abinit_gene_0_1_mRNA_1 | IIIb | SchWRKY085       | g10699.t1       | IIIb |
| -                  | -                    | -                | -              | SchWRKY086a      | g9512.t1       | III |
| -                  | -                    | -                | -              | SchWRKY086b      | g9513.t1       | III |
| Table 1. List of ScWRKYs and SchWRKYs with the locus ID and the division in Groups. | 752 (ScWRKY87), and that of SchWRKYs from 123 (SchWRKY21) to 744 (SchWRKY3) (Supplementary Table 2). The exon-intron organization of our WRKY genes was examined to gain more insight into the evolution of the WRKY family in potato. As shown in Supplementary Table 2, all ScWRKY genes possessed from one to eight exons. A similar trend was observed in S. chacoense. Concerning the genomic localization of WRKY genes, due to the unavailability of S. commersonii physical map, we plotted genes only on S. chacoense chromosomes using the Phenogram online tool (http://visualization.ritchielab.org/phenograms/plot) (Figure S1). Out of 84 SchWRKY genes identified, 83 were mapped. As represented in Figure S1, most of the genes were located on chromosome 3 (11 genes; 13.1%), followed by chromosome 5 (10; 11.9%), Unknown (8; 9.5%) and 2 (7; 8.3%). A total of 25 SchWRKY genes (5 on each chromosome) were localized on chromosomes 7 to 12, whereas no one was mapped on chromosome 11. Expression patterns of WRKY genes in S. commersonii and S. chacoense. To explore the expression of WRKY genes, we analyzed and calculated the RNA sequence data available for leaf, flower and tuber in both species (Figs. 2a and 2b). The heat-map based expression profiles of ScWRKYs (Fig. 2a) and SchWRKYs (Fig. 2b) revealed their dynamic and differential expression in various tissues and that the range of expression |
varied among the two species. In *S. commersonii*, 21 (26.5%) *ScWRKY* genes (01, 14, 15-like, 15-like_2, 21, 30, 39, 58-like, 60, 61, 62, 62-like, 63, 66-like, 67, 81, 84_like, 85, 86, 88 and 89) showed very low or undetectable expression (FPKM values from 0 to 0.5) in all studied tissues, while 16 (20.2%) genes (18, 23, 03, 48, 87, 08, 11, 47, 79, 10, 51, 45, 05, 06, 12 and 49) were highly expressed (FPKM > 5) in all tissues. Some of the remaining genes showed tissue specificities. *ScWRKY*002, *ScWRKY*013 and *ScWRKY*017 were highly expressed only in flower, and *ScWRKY*042 and *ScWRKY*080 only in leaf, while no tuber specific *ScWRKYs* were identified. In *S. chacoense*, 21 (25%) *SchWRKY* genes (4, 14, 15, 16, 21, 34, 56, 57, 60, 61, 62, 63, 64, 67, 69, 81, 83, 86, 87a, 88 and 89) showed no expression in all considered tissues, while 42 (50%) were overexpressed in all tissues. Concerning the remaining genes, nine leaf-specific (*SchWRKY*001, *SchWRKY*017, *SchWRKY*024, *SchWRKY*027, *SchWRKY*043, *SchWRKY*059, *SchWRKY*073, *SchWRKY*077 and *SchWRKY*085) and three flower-specific genes (*SchWRKY*028, *SchWRKY*030 and *SchWRKY*087b) were identified. As is the case of *S. commersonii*, no tuber specific *SchWRKYs* were found.

Four *ScWRKY* genes (*ScWRKY*016, *ScWRKY*023, *ScWRKY*045 and *ScWRKY*055) distributed in different Groups were selected to further investigate WRKYs behaviour in response to biotic (wounding) and abiotic (PVY and *P. carotovorum*) stressors using qRT-PCR (Fig. 3). The expression trend of our WRKYs was variable among and during treatments. In particular, wounding stress caused *ScWRKY*023 and *ScWRKY*045 overexpression during the whole treatment and *ScWRKY*055 overexpression at 4- and 6-hours post treatment (hpt). As for viral infection response, *ScWRKY*016 and *ScWRKY*045 were always downregulated, while the other genes were upregulated only at one of the five hpt. The bacterial inoculation with *P. carotovorum* did not activate *ScWRKY*016.

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**Figure 1.** Phylogenetic analysis WRKY proteins in *S. commersonii*, *S. chacoense* and seven representative proteins of *Arabidopsis*. Multiple sequence alignments of WRKY amino acid sequences were performed using ClustalX, and the phylogenetic tree was constructed using MEGAX by the Maximum Likelihood (ML) method and 1000 bootstrap replicates. The tree was divided into seven phylogenetic subGroups and distinguished by colours: dark purple for Group I, light blue for subGroup Ia, orange for subGroup Ib, light purple for subGroup Ic, dark blue for subGroup IId, green for subGroup Ile, red for Group III. The bootstrap values were ≥85%.
and ScWRKY045, while the other two genes were upregulated at 2- and 6-hpt. Given the involvement of WRKYs in several biological processes, we wondered whether they might play roles under other stresses. Since WRKY expression data on wild potato species exposed to any stress are not available, we retrieved WRKYs RPKM values.
from *S. tuberosum* experiments involving several treatments and stressors. As shown in Figure S2, the transcription of most WRKY genes was affected by various treatments. Only StWRKY61 to StWRKY67 did not change their transcriptional activity upon stress. The late blight infection did not perturbate the expression of StWRKYs. *StWRKY023, StWRKY044, StWRKY054* and *StWRKY055* increased their expression following mannitol treatment, whereas ABA, IAA and GA3 hormonal treatments affected the transcriptional activity of 3 (*StWRKY027, StWRKY028* and *StWRKY046*), 1 (*StWRKY035*) and 4 (*StWRKY023, StWRKY054, StWRKY068, StWRKY070*) *S. tuberosum* WRKYs, respectively. BABA and BTH treatments induced an overexpression of 18 and 15 StWRKYs respectively, of which *StWRKY042, StWRKY075, StWRKY078* and *StWRKY080* were in common. Concerning heat stress, 12 StWRKYs were overexpressed. Finally, under in- vitro culture conditions, 10 StWRKYs were overexpressed in shoots and one (*StWRKY004*) in roots.

**In silico protein interaction network of selected ScWRKYs and SchWRKYs.** A network of interaction was studied for WRKYs showing either tissue-specific or stress-induced expression (Figure S3a and S3b). The *S. commersonii* flower-specific expressed WRKY002 formed a node with the anthocyanins and cell differentiation regulatory proteins. STRING analyses provided evidence that ScWRKY002 interacts, among the others, with JAF13 and TTG1, two well-characterized potato anthocyanins bHLH and WD40 TFs. Both the leaf-specific expressed ScWRKY042 and ScWRKY080 formed a cluster of interaction with a Leucine Rich Repeat (LRR) protein (an evolutionarily conserved protein associated with innate immunity in plants). The two wounding-responsive

**Figure 3.** Expression RT-qPCR analysis of selected ScWRKY genes under abiotic and biotic stresses: wounding, PVY and *P. carotovorum*. For each stress the same time course of 0, 1, 2, 4, 6 hours post treatment was considered. The y-axes represent the mean relative expression normalized against non-treated plants for wounding stress and water-treated plants for PVY and *P. carotovorum* inoculations. Standard deviation values are shown.
ScWRKY023 and ScWRKY045 established two independent nodes of interaction. The former set a cluster with "Wound-responsive Apetala2 like factor 2 (WRFL2)" (annotation for transcript PGSC0003DMT400021314 on SpudDB database), while ScWRKY045 interacted with a cluster of proteins linked to a class of glycosyltransferase. Concerning S. chacoense, ScWRKY030 (found to be flower-specific) interacted directly with eIF2B_5, a key protein involved in mRNA translation mechanisms. On the counterpart, the leaf-specific SchWRKY107, SchWRKY043, SchWRKY059 and SchWRKY077, together with the flower-specific SchWRKY028, showed the same interaction with LRR proteins already described for ScWRKY042 and ScWRKY080.

Discussion
Due to its importance in the regulation of several processes in plants, the WRKY family has been studied in more than 60 plant species. In Solanaceae, data are available in some important crops, such as S. tuberosum (79, 82 and 8112 WRKYs), S. lycopersicum (83 WRKYs)43 and S. melongena (50 WRKYs)45. However, no information is available on the number and structural variability of WRKY TFs in Solanaceae wild species, which represent an important reservoir of genetic variation for breeding. This study was set up with the aim to profile WRKY encoding genes in S. commersonii and S. chacoense, two noteworthy tuber-bearing potato species used in potato breeding programs.20–45.

Structural analysis of ScWRKYs and SchWRKYs revealed interspecific diversification. The recently published genome annotation of S. commersonii13 and S. chacoense14 enables a comprehensive investigation of the WRKY family. We detected 79 and 84 genes encoding putative WRKY TFs in S. commersonii and S. chacoense, respectively. These results indicate that, compared to the cultivated potato26, S. commersonii possesses a lower number and S. chacoense a higher number of WRKY genes. Both species displayed a number of WRKYs greater than that of barley (45)46, castor bean (58)46, cucumber (55)48, rapeseed (43)49 and grapevine (59)46, and lower than that of cotton (120)50, maize (136)52, soybean (131)53 and rice (100)52. From this comparison, it appears that the number of WRKY encoding genes is not proportional to the genome size of the respective plant species, as also reported by Waqas et al.54. ScWRKY and SchWRKY proteins were primarily divided into three main phylogenetic Groups with Group II further classified into five subgroups (IIa-Ile). Most of WRKYs found in the two wild species belonged to Group II and this is in line with results obtained in S. tuberosum32. As known, WRKY proteins are characterized by one or more WRKY domain. In this study, we found that ScWRKYs and SchWRKYs had either one or two WDS. Interestingly, two ScWRKYs (ScWRKY010 and ScWRKY002) carried three WDS. This might be the result of the acquisition of a WRKY domain during evolution, supporting findings of Aversano et al.19 and Esposito et al.31,56, who reported that S. commersonii prosper lineage-specific segmental duplications during evolution. Not only WDS number, but also WDS structural diversifications identified in S. commersonii and S. chacoense might be the consequence of mutations during the process of evolution. Almost all ScWRKYs and SchWRKYs have the highly conserved heptapeptide WRKYGQK motif, except for eight variants. Among them, WGKYTGQK of ScWRKY014, WRWKGC of ScWRKY006, WNYKQGQK of SchWRKY014 and WKHHGKS in SchWRKY057 were not found in any other species. On the counterpart, the remaining variants were identified also in S. tuberosum32, S. lycopersicum46, H. vulgare22 and C. annum56. Zhou et al.46 hypothesized that these variations may change the DNA targets' binding specificity. The structural diversity has been investigated also at the genome level through the identification of exons and introns. As reported by Shiu and Bleecker22, this highlights events of diversification and neo-functionalization of WRKY genes. In contrast to findings by Wang et al.61, our results did not reveal a conservation of gene structure among the members of the same Group, even though they allowed the discrimination of eight intron-lacking WRKYs (two ScWRKYs and six SchWRKYs). This is in agreement with results reported in the cultivated potato, where StWRKY23 and StWRKY24 had no introns. Lynch et al.82 hypothesized that the intron turnover can be the result of reverse transcription of the mature mRNA followed by homologous recombination with intron-containing alleles.

Identification of tissue-specific and stress responsive WRKYs in wild potatoes. WRKY TFs have been found to play important roles under abiotic stresses, such as drought8, heat63, wounding50, and biotic constraints, such as bacteria19 and viruses64. Tissue-specificity of WRKY genes has also been highlighted in different crops, such as pepper58, cotton65 and soybean66, elucidating their role in developmental and functional processes. Our study investigated for the first time the stress response and tissue-specificity of WRKY genes in two wild potato species. Six and 11 WRKY genes were identified as flower- and leaf-specific, respectively. Zhang and collaborators73 considered that the known protein-protein interaction network can provide important clues to better understand gene expression regulation. Basing on this, we investigated the interactome of WRKY TFs in S. commersonii/SchWRKYs and genes of various pathways. From our analyses, interesting observations and different clues for future functional studies have emerged. For example, ScWRKY002 could be in some way involved in anthocyanin activation in flowers of S. commersonii: it interacts with anthocyanin BHLHs, and the flower of this wild species strongly accumulates anthocyanins.44,67 Previous studies reported that some WRKYs can be involved in the coordination of multiple biological processes. For example, AWRKY33 regulates disease resistance, NaCl tolerance and thermotolerance28–31, while GHWRKY40 modulates tolerance to wounding stress and resistance to R. solanacearum43. This suggests that some WRKY proteins provide important nodes of crosstalk between different physiological processes. However, the putative members of WRKY family and their possible roles in signalling crosstalk are still barely known. To the authors' best knowledge, no expression data are available on ScWRKYs and SchWRKYs; by contrast, StWRKYs have previously received attention. Among them, only Shahzad et al.71 and Yogendra et al.72 found StWRKY10 (PGSC0003DMP400029302) and StWRKY020 (PGSC0003DMP400028763) to be active in P. infestans-potato interaction. Consistently with these data, our results indicated that the same genes increased their expression after BABA treatment, known to
Confer protection against several biotic threats. Furthermore, we focused our attention on a group of proteins (ScWRKY016, ScWRKY023, ScWRKY045 and ScWRKY055) which were reported to be stress-responsive. For these genes, we tested the transcriptional activity of wild S. commersonii alleles after wounding and bacterial infection and investigated on their direct ortholog expression on various treatments. Among them, StWRKY016, StWRKY045 and StWRKY055 appeared to be required by plants to face damages by heat stress, while StWRKY023 was reported to be active under mannitol and GA3 treatments as well as drought stress. Our results suggested that the wild alleles of ScWRKY023 and ScWRKY045 might represent promising candidates for multiple stress responses as they are leaf-specific and constantly expressed after wounding in S. commersonii but not in the cultivated potato. In addition, WRKY023 is also induced by bacterial infection and it is suggested to interact with both a WRAF2-like protein and with the LRR mediated immunity system.

Conclusions
The present study identified 79 and 84 genes encoding putative WRKY TFs in S. commersonii and S. chacoense, respectively. Their protein structure and data from the comparative analyses suggested an interspecific variability of WRKY genes. Most of them were up-regulated under stress conditions and across different tissues, hinting a possible role in the cross-talk between plant and environmental cues in potato species. Taken as a whole, these analyses will help to hasten the determination of the function of WRKY TFs especially in response to biotic and abiotic stresses. Candidate ScWRKY and SchWRKY genes identified here can be employed in potato breeding programs.

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**Author contributions**
V.C. and E.S. performed the analyses, processed the experimental data, interpreted the results and contributed to figure designing and manuscript writing. G.R., A.D. and Z.A. conducted experiments. V.D. provided critical feedback and helped shape the research, analysis and manuscript. A.R. and C.D. conceived the idea of study, coordinated the work and contributed to results interpretation and manuscript writing. All authors read and approved the manuscript.

**Competing interests**
The authors declare no competing interests.

**Additional information**
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