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The Analysis of *Pinus pinaster* SnRKs Reveals Clues of the Evolution of This Family and a New Set of Abiotic Stress Resistance Biomarkers

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Abstract: Climate change is increasing the intensity and incidence of environmental stressors, reducing the biomass yields of forestry species as *Pinus pinaster*. Selection of new stress-tolerant varieties is thus required. Many genes related to plant stress signaling pathways have proven useful for this purpose with sucrose non-fermenting related kinases (SnRK), conserved across plant evolution and connected to different phosphorylation cascades within ABA- and Ca^{2+}-mediated signaling pathways, as a good example. The modulation of SnRKs and/or the selection of specific SnRK alleles have proven successful strategies to increase plant stress resistance. Despite this, SnRKs have been barely studied in gymnosperms. In this work *P. pinaster* SnRK sequences (PpiSnRK) were identified through a homology- and domain-based sequence analysis using *Arabidopsis* SnRK sequences as query. Moreover, PpiSnRKs links to the gymnosperm stress response were modeled out of the known interactions of PpiSnRKs orthologs from other species with different signaling complexity. This approach successfully identified the pine SnRK family and predicted their central role into the gymnosperm stress response, linking them to ABA, Ca^{2+}, sugar/energy and possibly ethylene signaling. These links made the gymnosperm kinases promising candidates into the search for new stress resistance-related biomarkers, which would be useful into future breeding strategies.

Keywords: stress; forestry; *Pinus pinaster*; SnRK

1. Introduction

The ever-increasing intensity of environmental stressors and the changes into their seasonal incidence, both associated to climate change, have a negative and global impact in plant biomass yields [1–4]. *Pinus pinaster* is a gymnosperm species with a key environmental and forestry relevance into the northwestern Mediterranean area, where it is used for afforestation due to its fast growth, high phenotypic plasticity and stress tolerance, soil stabilization capacity and the quality of its timber and oleoresins [5,6]. Moreover, this species is also a good example of the climate-associated reductions in plant biomass yield related to regional climatic changes, increasing abiotic—heat and drought—and biotic stress pressure, which are also a main constraint for other *Pinus* species [7–10].

Multiple efforts have been started globally to avoid climate change effects over plant production directed to the use of better management strategies and the selection of more stress tolerant and productive plant varieties [3]. Despite new stress tolerant varieties are required to cope with the climate change effects and/or increase productivity, their generation faces the complexity of plant traits as stress resistance, growth speed and wood quality. Compared to crops, in tree species and particularly in gymnosperms as *P. pinaster* the complexity problem add up to the large and poorly characterized

genomes. These large sizes complicate the characterization of forestry desirable traits since they are generally polygenic. Many pathways aimed at these stress- and biomass-associated traits, as the ones involving abscisic acid (ABA), reactive oxygen species (ROS), jasmonates/salicylates and Ca^{2+}, have been largely characterized in herbaceous plant models as *Arabidopsis thaliana*, and the modulation of genes within these routes improves stress tolerance in many plant species [11–13], however the knowledge in pine is scarce. Therefore, the characterization of these pathways in gymnosperms and their role into the modulation of these species' agronomical traits will contribute to better understand the species stress response and potentially identify new biomarkers for breeding.

Sucrose non-fermenting related kinases (SnRK) are central and conserved elements in the phosphorylation cascades within ABA- and Ca^{2+}-mediated plant stress signaling pathways [14]. This kinase family divides into three functionally divergent subfamilies, namely SnRK1, SnRK2 and SnRK3. The SnRK1 subfamily, conserved across eukaryotes, links central metabolism with stress response to modulate the cell responses to energy stress [15]. SnRK1 kinases are part of protein complexes involving different regulatory subunits, namely β, γ and βγ where β modulates the kinase substrate specificity and its cell location [16], and γ acts as a sensor of the ADP-AMP levels monitoring the cell energy status [17]. βγ is a plant specific subunit functionally equivalent to the animal γ subunit, as plant γ orthologs do not interact with SnRK1 [18]. SnRK2 and three subfamilies are also Plantae-specific elements, which share with SnRK1 a common Ser/Thr kinase domain. This kinase domain is followed in SnRK1 by a UBA and KA domains, which allow its interaction with their different regulatory subunits. Conversely, the kinase domain is followed by different regulatory domains in subfamilies 2 and 3. Regulatory domain I follows the kinase domain into all SnRK2 and is related to their activation by osmotic stress, while the kinase domain of all SnRK3 is followed by a FISL/NAF domain, which allows the interaction of SnRK3 kinases with Ca^{2+} signaling [14]. Some SnRK2 have a second regulatory domain (domain II) allowing their modulation by ABA. Arabidopsis SnRK2 sequences could be organized by their ABA sensitivity into the groups SnRK2 I (ABA insensitive), SnRK2 II (low ABA sensitivity) and SnRK2 III (high ABA sensitivity) where only group II and III sequences contain the ABA regulatory domain II [13].

SnRK kinases participate in the stress response both through the direct modulation of stress response effectors as ion channels, and the regulation of transcription factors—bZIP family—and epigenetic modulation mechanisms such as the SWI/SNF complex, which in turn control the expression of broad stress-related gene groups. The modulation of the expression of the SnRKs and/or their related elements as bZIP transcription factors through the use of genetic engineering or approaches exploiting their natural variation enhances stress resistance into both herbaceous species as *A. thaliana* and rice, and tree species as poplar [14,19,20]. Moreover, SnRKs are linked to the modulation of different agronomical traits under stress including wood quality. SnRK1, involved in sugar and energy signaling, modulates the flux of sucrose towards cellulose [21]. Furthermore, other SnRK related elements and signalers are also involved in xylogenesis. Poplar genes related to wood formation are enriched in bZIP binding motifs [22] and ABA abundance changes correlate with stress effects on wood structure [23]. Therefore, *P. pinaster* SnRK orthologs as probable ABA and bZIP intermediaries in the gymnosperm and possible links between the pine stress response and its wood quality are interesting targets in this species' enhancement strategies.

The description of the *P. pinaster* SnRKs, thereafter PpiSnRKs, will allow the identification of their orthologs in species with better characterized stress response systems as *A. thaliana*. These species would contribute first to predict and then to characterize the function of the pine kinases. Model species as *A. thaliana*, hereafter *Arabidopsis*, have already been used for the validation of candidate genes from tree species [24]. Moreover, the identification of the PpiSnRK orthologs into related species with close but simpler stress response systems would further ease the characterization of the pine kinases. *Amborella trichopoda*, an ancient angiosperm closer to gymnosperms than Arabidopsis, *Selaginella moellendorffii*, a non-seed plant, and *Marchantia polymorpha*, a non-vascular plant, (hereafter *Amborella, Selaginella* and *Marchantia*) are, along the less evolved chlorophyte microalgae

species, promising species for this purpose. The modulation of the PpiSnRK orthologs of these species and/or their substitution for their *P. pinaster* counterparts would allow the characterization of the *P. pinaster* PpiSnRKs, reducing the required time-consuming transformation and/or selection strategies in the tree species. Moreover, the identification of the orthology links with these plant and microalgae species, with different positions in relation to the last microalgae-plant common ancestor, would trace down the evolution of the complex plant SnRK families and evidence many of the specific features of the gymnosperms SnRK families.

The known relevance of SnRKs for the enhancement of stress resistance and biomass production in plants, and the lack of knowledge of this kinase family in gymnosperms, has motivated the description of the SnRK family of the gymnosperm species *P. pinaster*. PpiSnRKs were described, and their interactions modeled through an approach based on sequence analysis and protein interaction prediction, comparing the pine SnRKs with their orthologs from different plant species. These approaches would give hints about the evolution of SnRK family in gymnosperms and predict the specific role of the identified PpiSnRKs, which would ultimately contribute to the selection of specific ones related to some traits desired in forestry. These selected PpiSnRKs and or their related proteins could be characterized in simpler plant systems and then possibly used as biomarkers to drive future *P. pinaster* breeding programs.

2. Materials and Methods

2.1. Description of the P. Pinaster PpiSnRK Family

P. pinaster transcriptome and proteome [25] available at Plaza [26] were used for the identification of the gymnosperm SnRK orthologs to the *Arabidopsis* SnRK family [14]. All of the *Arabidopsis* sequences of the family were used as query into BLAST- and BLASTP-based searches against *P. pinaster* transcriptome and proteome using the default NCBI BLAST and BLASTP parameters. Only hits with e-values lower than e^{-25} were considered for further analyses. Non-SnRK sequences were filtered out from homology results through the analysis of their domain composition using the version 75.0 of the Inter Pro Scan database [27]. Only *P. pinaster* sequences with the *Arabidopsis* SnRKs canonical domain composition—including Ser/Thr kinase domains (PTHR24343, PTHR43895), UBA (IPR015940), KA1/ αCTD (IPR001772), Immunoglobulin E-set (IPR014756), AMPK glycogen binding subunit (IPR032640), ASC (IPR006828), Immunoglobulin E-set (IPR014756), CBS (IPR000644) and NAF/FISL (IPR018451) domains (Table S1)—were maintained for further analyses as previously specified [28]. *Pinus pinaster* PpiSNRK transcript sequences were used for the identification of possible PpiSNRK isoforms. PpiSNRK transcripts were aligned with MUSCLE alignment algorithm using default parameters, considering a group of aligned transcripts isoforms when sharing 3 and 5′ UTR regions. *Pinus pinaster* PpiSNRK transcripts [25] and *P. taeda* [26] transcript orthologs to the Arabidopsis SnRK sequences were used for the curation of the incomplete and fragmented *P. pinaster* PpiSnRK protein models. The *P. pinaster* and *P. taeda* transcript sequences were translated, and the resulting protein sequences aligned using the M-Coffee consensus alignment method with default parameters to their respective PpiSNRK protein models for their curation.

2.2. Alignment of SnRK Sequences

PpiSnRK sequences were aligned with other SnRK sequences belonging to *Arabidopsis* and different microalgae species [28] (Table S2) using the M-Coffee consensus alignment method with default parameters [29]. Alignment was filter-curated through the Transitive Consistency Score algorithm (TCS) using default parameters [30]. Curated alignment distances were used in the generation of a maximum likelihood (ML) tree whose consistency was evaluated through the Transfer Bootstrap Expectation score (TBE, 500 replicates) included in the FastTree workflow within the Booster platform [31] due to the high number of involved sequences.

SnRK2 sequences from microalgae and different plant species including *P. pinaster* PpiSnRK2, *Arabidopsis, Amborella* and *Selaginella* (Table S2) were aligned using M-Coffee multiple alignment

method with default parameters [29]. Different alignments were made by changing the included plant species set to test the resulting trees topology consistency. TCS weight-curated alignment distances were used to build rooted ML trees whose consistency was evaluated through Felsestein's bootstrap (FBP, 500 replicates) into the PhyML-SMS workflow within the booster platform [31].

2.3. Bioinformatic and Statistical Analyses

All the procedures for the identification and classification of *P. pinaster* PpiSnRK were performed locally employing the bioinformatics suite Geneious v7 (Biomatters Inc.), with the exception of Inter Pro Scan [27] searches, M-Coffee alignments and TCS-based alignment curation, and ML trees construction that were respectively performed at the European Bioinformatics Institute (ebi.ac.uk), T-Coffee (tcoffee.crg.cat) and booster (booster.pasteur.fr) websites.

Protein–protein functional interactions between the pine PpiSnRK2 sequences were inferred using the STRING v11 [32] database. As *P. pinaster* or other gymnosperms were not available at the interaction database, PpiSnRK2 orthologs from related STRING-available species as *Amborella*, *Selaginella* and *Arabidopsis* were separately used as query into the STRING website (string-db.org). Three networks were made (one for each species) showing high confidence (over 0.7 STRING interaction score), known and predicted associations between proteins.

3. Results

3.1. Identification of the P. pinaster SnRK Family Members

The search of the *Arabidopsis* SnRKs [14] in *P. pinaster* transcriptome and proteome by using BLAST and BLASTP revealed a large protein set including SnRK homologs and non-SnRK sequences sharing the conserved Ser/Thr kinase domain. The analysis of the domain composition of these proteins allowed to unequivocally distinguish the PpiSnRK sequences attending to the exclusive SnRK domains surrounding the conserved kinase domain and/or the specific features of their kinase domain. The combination of BLAST-based search and domain analysis over *P. pinaster* available sequence data resulted in the confident identification of 7 PpiSnRK1-associated regulatory subunits and 45 PpiSnRK kinases belonging to the subfamilies 1, 2 and 3, including multiple isoforms (Table S1).

The M-Coffee alignment tree of the identified *P. pinaster* kinases with their orthologs in *Arabidopsis* and different microalgae species (Table S2, Figure 1) together with the description of the pine PpiSnRK domain structure were the bases for defining the three catalytic clusters of this family in pine, namely PpiSnRK1, PpiSnRK2 and PpiSnRK3 (Figure 1, Table S1). A fourth cluster, described by the domain analysis, contained the PpiSnRK1-associated regulatory subunits (Table S1). PpiSnRK1 cluster included the *P. pinaster* SnRK1 ortholog (PpiSnRK1; Ser/Thr kinase (PTHR24343), UBA (IPR015940) and KA1/ αCTD (IPR001772) domains) and the SnRK1-like sequence (PpiSnRK1-L; Ser/Thr kinase (PTHR24346)) along their orthologs in other species (Figure 1, Table S1). *Arabidopsis* SnRK1/AKIN10 was the closest sequence to *P. pinaster* PpiSnRK1 and PpiSnRK1L clustered along the *Chlamydomonas reinhardtii* and *Volvox carteri* SnRK1 like sequences within this group (Figure 1). The fourth and PpiSnRK1-associated cluster included the non-catalytic regulatory subunits of the PpiSnRK1 complex: PpiSnRKβ1, β2 (Immunoglobulin E-set (IPR014756) AMPK glycogen binding subunit (IPR032640) and ASC (IPR006828) domains), PpiSnRKβγ1, βγ2 (Immunoglobulin E-set (IPR014756) and CBS (IPR000644) domains) and related PpiSnRKγ1, γ2, γ3 (CBS domains (IPR000644); Table S1).

PpiSnRK3 cluster (Ser/Thr kinase (PTHR43895) and NAF/FISL (IPR018451) domains) was the biggest sequence group in the tree (Figure 1, Table S1), mostly represented by the *Arabidopsis* and *P. pinaster* elements. SnRK3 sequences from less evolved microalgae species as *Coccomyxa subellipsoidea* CsCKIN3 and *Chlorella variabilis* CvCKIN3 group together (PpiSnRK3 cluster, Group 1), placed at the base of this cluster followed by four more groups containing exclusively *Arabidopsis* and *P. pinaster* sequences (PpiSnRK3 cluster, Groups 2-5; Figure 1). The closest plant SnRK3 group to the microalgae specific Group 1 was Group 2 and contained *Arabidopsis* SnRK3.11 and SnRK3.13, and *P. pinaster*

PpiSnRK3.3 and PpiSnRK3.4 (Figure 1). *Arabidopsis* SnRK3.11 or SOS2 is involved into the SOS salt stress signaling pathway [33].

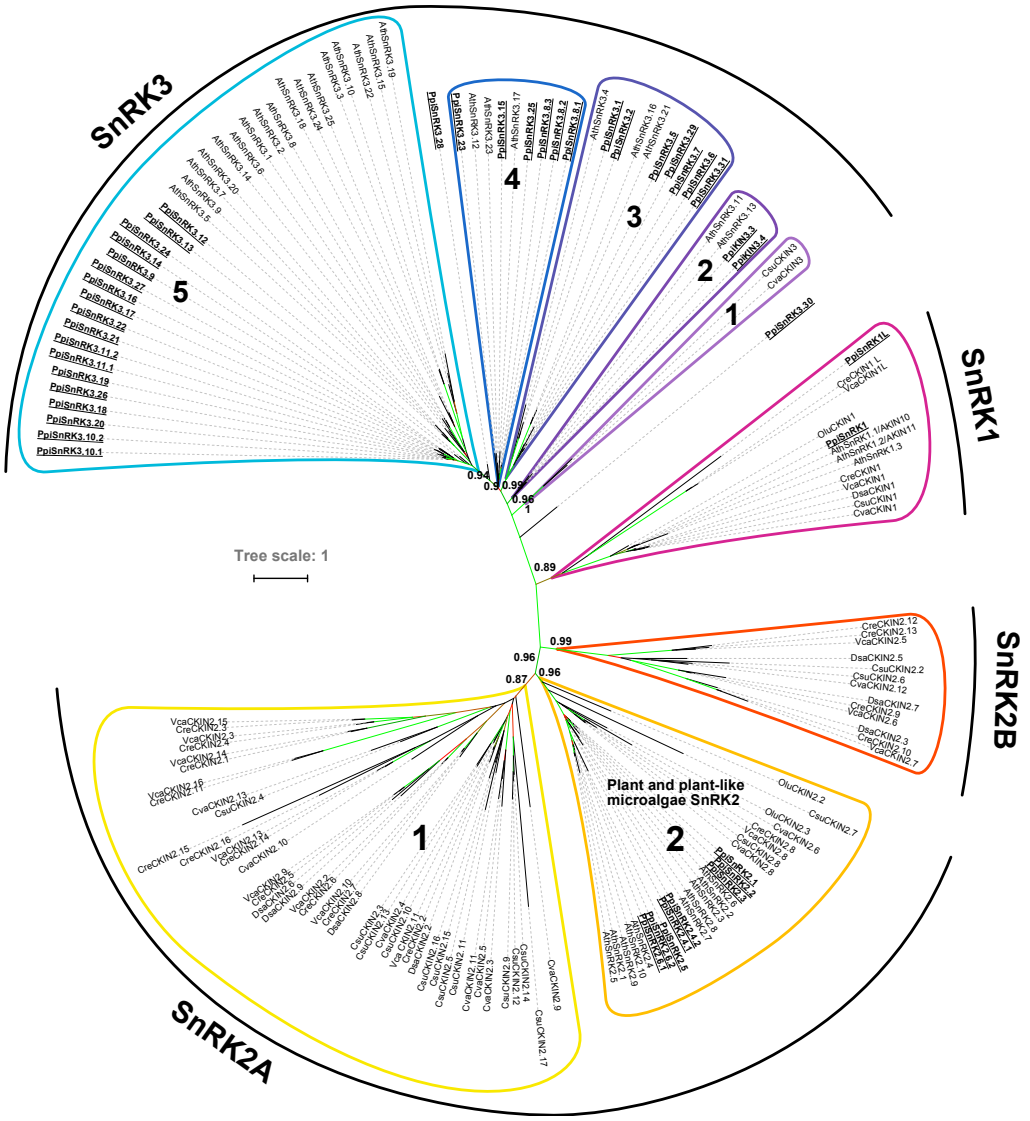

Figure 1. Unrooted maximum likelihood (ML) tree of *Pinus pinaster* (PpiSnRK) and *Arabidopsis thaliana* (AthSnRK) SnRK sequences along their orthologs into different microalgae species—CKIN—including *Chlamydomonas reinhardtii* (CreCKIN), *Volvox carteri* (VcaCKIN), *Dunaliella salina* (DsaCKIN), *Chlorella variabilis* (CvaCKIN), *Coccomyxa subellipsoidea* (CsuCKIN) and *Ostreococcus lucimarinus* (OluCKIN) SnRK sequences. Tree branches are colored according to their TBE bootstrap values. Branches with TBE values below 0.8 are black, while these above 0.8 TBE are colored from red (0.8) to light green (1). Tree divided the sequences into the tree SnRK families (SnRK1, 2, 3). SnRK1 subfamily was divided into SnRK1 like and SnRK1 sequences, SnRK3 sequences was divided into five different subgroups and SnRK2 sequences were divided between SnRK2A and SnRK2B. *P. pinaster* PpiSnRK2 sequences were all included into the SnRK2A group along *Arabidopsis* SnRK2s.

The third cluster, PpiSnRK2 sequences (Ser/Thr kinase (PTHR24343) domain), was divided into SnRK2(A) and (B) clusters as previously described [28] with all the *P. pinaster* PpiSnRK2 and *Arabidopsis* SnRK2 sequences falling into the SnRK2(A) cluster (Figure 1, Table S1). SnRK2(A) also included a small group of plant-like microalgae SnRK2s conforming the Group 2 of SnRK2(A) (Figure 1). *P. pinaster* sequences as PpiSnRK2.5, and PpiSnRK2.6 isoforms were confidently clustered along *Arabidopsis*

SnRK2.1, 2.4, 2.5, 2.9 and 2.10 sequences, but the tree failed to cluster remaining ones with any of the *Arabidopsis* SnRK2 sequences (Figure 1).

3.2. Description of the P. pinaster PpiKIN2 Subfamily Structure and Sequence Features

M-Coffee-based trees of *P. pinaster* PpiSnRK2, and other plant and microalgae SnRK2 orthologs confidently separated plant SnRK2 and microalgae CKIN2/SnRK2 (Figure 2) as the previous analysis (Figure 1). *Arabidopsis* SnRK2 sequences were confidently grouped into the three previously described groups, namely I (ABA insensitive), II (Low ABA sensitivity) and III (high ABA sensitivity) [34] into all SnRK2 trees (Figure 2). *Pinus pinaster* PpiSnRK2.5, 2.6.1 and 2.6.2, and PpiSnRK2.1, 2.2 and 2.3 fell within Groups I and III respectively into all tested tree topologies. Conversely, PpiSnRK2.4 isoforms did not cluster consistently with any of the previous groups (Figure 2). Most *P. pinaster* and *Amborella* (phylogenetically closer to pine than *Arabidopsis*) SnRK2 were consistently clustered within I and III Groups along the *Arabidopsis* sequences. On the other hand, the consistency of Group II was lower across trees and *Arabidopsis* SnRK2(II) sequences—SnRK2.7 and SnRK2.8—were the only constantly present in this branch (Figure 2). *Amborella* SnRK2(II) sequence only clustered with *Arabidopsis* SnRK2(II) sequences into one of the trees (Figure 2b), and *P. pinaster* PpiSnRK2.4 isoforms were either equidistant to all groups (Figure 2a,c) or to II and III groups (Figure 2b). *Selaginella* sequences had a basal position in the plant SnRK2 branch of the SnRK2/CKIN2 tree and none of its sequences were clustered within the previously defined groups (Figure 2c). Furthermore, built trees were unable to converge into a basal SnRK2 group (Figures 1 and 2).

The ABA-sensitive cluster III of SnRK2 was highly similar between *Arabidopsis* and *P. pinaster*. PpiSnRK2.1, 2.2 and 2.3 had highly conserved regulatory domains I and II (ABA box) after the characteristic SnRK Ser/Thr kinase domain, supporting their involvement in the gymnosperm ABA signaling (Figure 3c). *P. pinaster* PpiSnRK2.5, 2.6.1 and 2.6.2, within the Group I SnRK2, also conserved a regulatory domain I after the Ser/Thr kinase domain (Figure 3a). PpiSnRK2.4 isoforms conserved the regulatory domain I, which was followed by a C-terminal sequence with features observed in the regulatory domain II of Group II and III SnRK2 sequences (Figure 3b).

Selaginella SnRK2 homolog sequences had a basal position in the SnRK2 tree and did not cluster with any of the defined SnRK2 groups (Figure 2c). In spite of this, these sequences showed analogy to *Arabidopsis* ABA sensitive II and III SnRK2. *Selaginella* SnRK2 had a conserved Domain I followed by an aspartate-rich domain II-like. These acidic aspartate residues are characteristic of the SnRK2s terminal domains of Groups II and III (Figures 2 and 3, Figure S1).

3.3. Arabidopsis, Amborella and Selaginella Orthologs to P. Pinaster PpiSnRK2 Are Connected to Stress Signaling and Metabolism Modulation

The *Arabidopsis, Amborella* and *Selaginella* orthologs to *P. pinaster* PpiSnRK2 were used to build three different STRING-based protein interaction networks from which a network outline was created pointing to the potential function of the pine PpiSnRK2 sequences (Figure 4, Figure S1). All the PpiSnRK2 orthologs across the different included species shared a connection to the SnRK1-related regulatory PpiSnRKβγ subunit and different starch modulatory enzymes, and to many PP2C and bZIP elements (Figure 4, Figure S1). In *Amborella* and *Arabidopsis* PP2Cs were functionally connected to the ABA receptors PYL/PYR/RCAR, which were also directly associated to these species group III SnRK2 kinases (Figure 4, Figure S1). The ABA receptor Mg chelatase (GUN5 or CHLH) and different ubiquitination-related elements were other conserved associations across the different species SnRK2 sequences. Despite the multiple connections conserved between the different species SnRK orthologs *Selaginella* and *Amborella* showed exclusive interactions. A protein phosphatase 2C and cyclic nucleotide-binding/kinase domain-containing protein (PP2C-L) was associated in the *Selaginella* and *Amborella* STRING networks (Figure 4, Figure S2b,c) with the conserved cluster of SnRK1, its associated regulatory βγ subunits and starch regulatory enzymes, while *Amborella* SnRK2(III) and *Selaginella* OST2.1 were connected to ethylene- and protein glycosylation-related elements respectively (Figure 4, Figure S1).

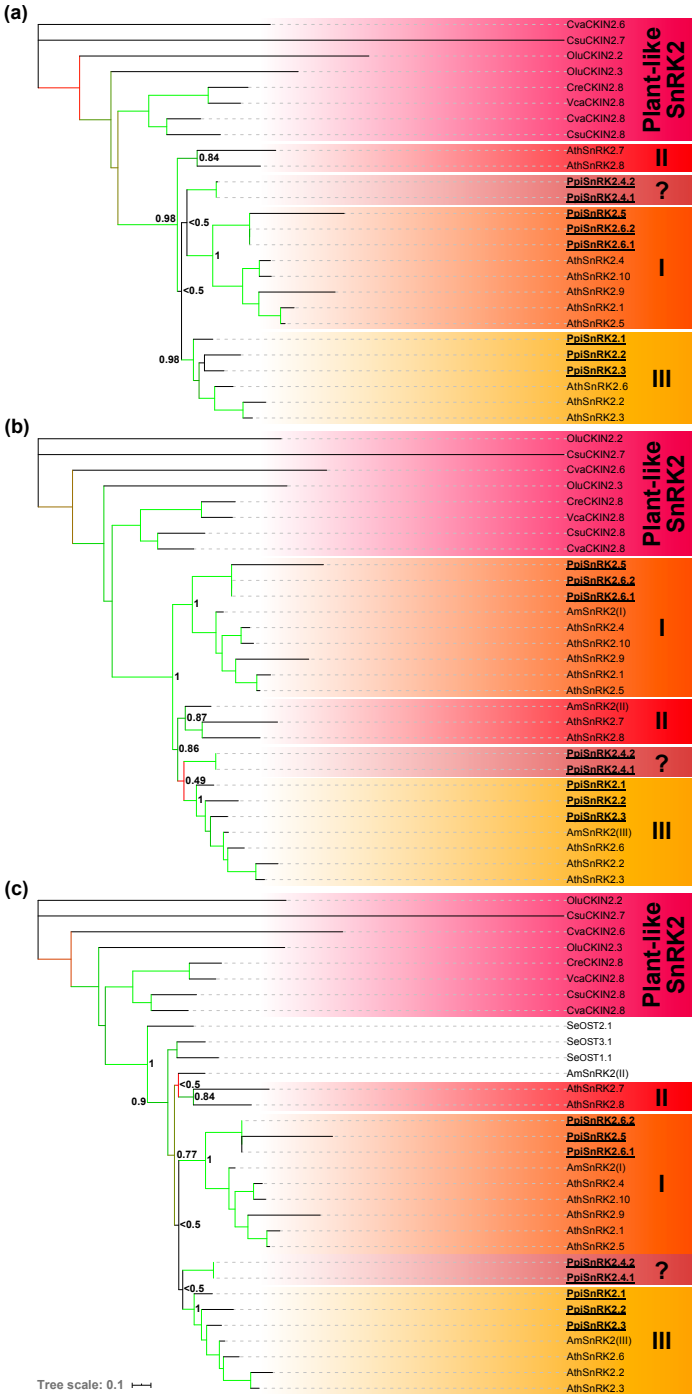

Figure 2. Rooted SnRK2 ML trees including SnRK2 orthologs from different sets of species along *Pinus pinaster* PpiSnRK2s: (**a**) *P. pinaster* (PpiSnRK2) and *Arabidopsis thaliana* (AthSnRK2) SnRK2 sequences along the plant-like SnRK2 orthologs (CKIN2) of different microalgae species including *Chlamydomonas reinhardtii* (CreCKIN2), *Volvox carteri* (VcaCKIN2), *Dunaliella salina* (DsaCKIN2), *Chlorella variabilis* (CvaCKIN2), *Coccomyxa subellipsoidea* (CsuCKIN2) and *Ostreococcus lucimarinus* (OluCKIN2); (**b**) same species and sequence set including *Amborella trichopoda* (AmSnRK2) SnRK2 orthologs and (**c**) same previous species and sequence set including *A. trichopoda* (AmSnRK2) and *Selaginella moellendorfii* (SeOST) SnRK2 orthologs. *Selaginella* and *Amborella* SnRK2 sequences, plant species with simpler SnRK2 subfamilies, were added to this SnRK2 sequence trees to evaluate the orthology links between the *P. pinaster* and these species SnRK2 subfamilies and their possible use as models for the characterization of the gymnosperm PpiSnRK2. Tree branches were colored according to their FBP bootstrap value, thus, branches with FBP values below 0.5 are black while these above 0.5 FBP are colored from red (0.5) to light green (1).

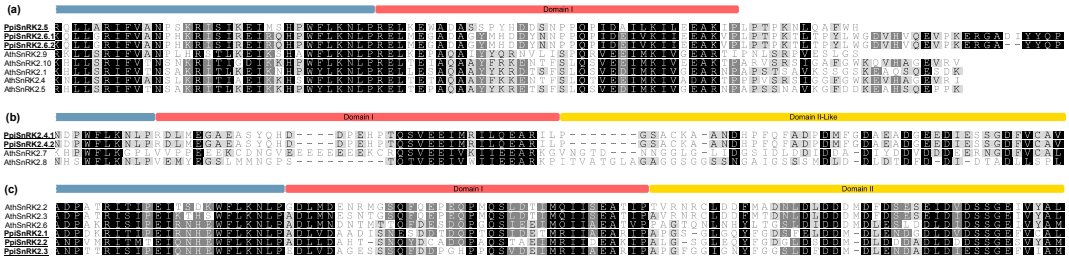

Figure 3. M-Coffee alignments of *P. pinaster* PpiSnRK2 and *Arabidopsis* SnRK2 sequences (AthSnRK2) focused on the C-terminal region of these kinases containing the regulatory domains I and/or II: (**a**) Group I SnRK2; (**b**) Group II SnRK2 and (**c**) Group III SnRK2. All pine PpiSnRK2 regulatory sequences showed high resemblance to the *Arabidopsis* sequences into their respective groups including PpiSnRK2.4 isoform, with conserved aspartate residues at positions corresponding with those of *Arabidopsis* SnRK2(II) sequences regulatory domain II.

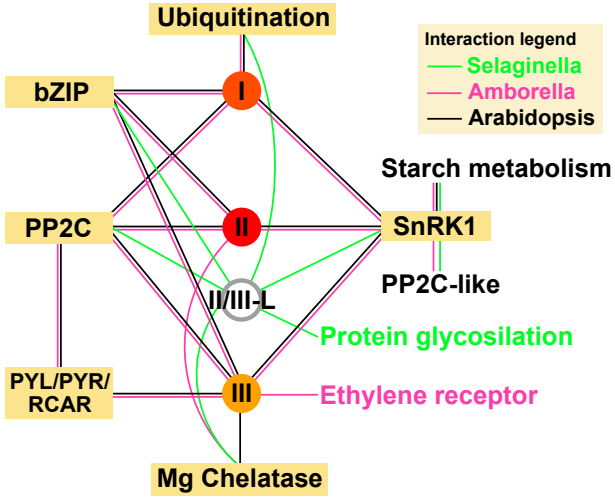

Figure 4. Network outline summarizing the known and predicted associations in the STRING networks for the *Arabidopsis* SnRK2s and their orthologs in *Amborella* and *Selaginella*. Most SnRK2 groups across the included species are related to the same processes and nodes highlighting bZIP transcription factors, ABA-related PP2C signaling and sensing and carbon metabolism. Some specific features also arose as the interaction of the *Amborella* SnRK2 III ortholog with an ethylene sensor or the interaction of the *Selaginella* sequences with elements related to the modulation of protein glycosylation. All connections shown in this outline are possibly conserved among PpiSnRK2 sequences taking into account the intermediate pine position between *Arabidopsis*, *Amborella* and *Selaginella*.

4. Discussion

Despite the great divergence between *P. pinaster* and *Arabidopsis*, the identified pine PpiSnRK and *Arabidopsis* SnRK families had similar size, structure, and sequence features, and potentially also similar interactions and roles. These parallelisms could be extended to the SnRK2 subfamilies of *Amborella*—a sequenced embryophyte close to the last gymnosperm-angiosperm common ancestor—and *Selaginella*, an also sequenced protovascular plant more ancient than gymnosperms. The observed similarities between these distant species point to the early origin of the SnRK family configuration and function known in *Arabidopsis* and other land plants [14,28]. Furthermore, the conservation evidences and the simpler SnRK families of these ancient model species motivated their election for the characterization of the pine PpiSnRK elements.

Pinus pinaster and *Arabidopsis* share large and similar SnRK3 subfamilies. A reduced group of these SnRK3 sequences could be considered founder as were close in the sequence tree to

microalgae SnRK3/CKIN3 (Figure 1). Interestingly, between the plant sequences included in this group are *Arabidopsis* SnRK3.11 or SOS2, a salt stress responsive kinase within the SOS pathway [33], osmostress-sensitive SnRK3.13 [35] and *P. pinaster* PpiSnRK3.3 and PpiSnRK3.4. This closeness points at once to the ancient (microalgae) origin of this pathway and by analogy to the possible involvement of both microalgae and *P. pinaster* PpiSnRK3.3 and PpiSnRK3.4 into salt and/or osmotic stress responses.

Many of the remaining *Arabidopsis* SnRK3 sequences, more distant from the microalgae ones, and clustering into the 3-5 SnRK3 clusters within the sequence tree (Figure 1) are involved in ABA stress signaling and expressed under certain developmental times and in specific tissues [36]. Thus, the diversification of the plant SnRK3 subfamily from the founder elements might have been related to the increase in structure and life cycle complexity of land plants and/or to their adaption to the more stressing land environment. Same explanation and function thus could be applied to the *P. pinaster* PpiSnRK3 sequences clustering along with the *Arabidopsis* ones in the 3-5 groups (Figure 1).

As SnRK3 and PpiSnRK3 subfamilies, PpiSnRK2 subfamily had a group division highly similar to *Arabidopsis* SnRK2. The SnRK2 subfamily sequence and structure similarity could also be expanded to *Amborella*, at the base of angiosperm group. Interestingly, although *Amborella* has a small SnRK2 family (three members), its members represent the three *Arabidopsis* SnRK2 groups. Again, the shared SnRK complexity and features between these distantly related species points to ancient duplication/divergence events—before angiosperm and gymnosperm division—but also to the conservation of these elements giving origin to the observed family similarity and pointing to their shared relevance and function. Besides this, the small size of *Amborella* SnRK2 family also points to gymnosperm and angiosperm independent SnRK2 duplication events. These independent duplication events could explain the observed grouping issues between elements sharing group II SnRK2 features as *Arabidopsis* SnRK2.7, SnRK2.8, *Amborella* AmSnRK2(II) and *P. pinaster* PpiSnRK2.4 isoforms.

On the other hand, *Selaginella*, a non-seed plant, has a simpler family structure exclusively composed by elements close to *Arabidopsis* SnRK2 II and III groups. The absence of ABA-insensitive group I SnRK2 sequences in *Selaginella* and their presence in *Amborella*, *Arabidopsis* and *P. pinaster* makes them the most recent SnRK2s. Group I SnRK2 would have originated at some point between vascular plant and seed plant origin. Moreover, the *Selaginella* SnRK2 subfamily points to the earlier origin of the ABA-sensitive SnRK2s and thus to the early involvement of ABA in SnRK2-based signaling pathways. This SnRK2(III) exclusive subfamilies have also been found in the moss *Physcomitrella patens* and charophyte algae supporting also the earlier origin of the ABA-sensitive elements [37]. Strikingly, *C. reinhardtii* CKIN2.8, a plant-like SnRK2, expression is not sensitive to exogenous ABA treatments [28].

Approaches including sequence and domain/motif analysis have been widely used along tree-based sequence clustering for the identification and description of many plant gene families, including the SnRK family. These strategies combined sequence-based methods with the evaluation of the identified sequences expression patterns in order to link them to particular stresses [28,38,39]. Other works [28,40] complemented this approach with the prediction of the proteins associated to the newly identified ones through databases such as STRING containing known relations between the orthologs to these elements. The involvement of apple bHLH transcription factors and *C. reinhardtii* CKIN/SnRK into their stress response system was predicted using this database. STRING predicted the link with ABA signaling of some of the identified apple bHLH genes and the link of *C. reinhardtii* CKIN/SnRKs to Ca^{2+} and PP2C signaling, and to the modulation of carbon and nitrogen metabolism [28,40].

STRING database was also chosen to predict the PpiSnRK2 sequences function and associations with the gymnosperm stress response system and metabolism using data from related plant and microalgae species with different positions within the Plantae species tree and different stress response system complexity. The overlap of the known and predicted STRING-based protein–protein interaction networks draw from the *P. pinaster* PpiSnRK2 orthologs in *Arabidopsis*, *Amborella* and *Selaginella* (Figure S2) outlined a network model of the possible associations of the *P. pinaster* PpiSnRKs with other stress related elements (Figure 4). These networks showed the conserved SnRK2 connections

with PP2C, bZIP transcription factors, ABA sensing/signaling and carbon metabolism modulation in *Arabidopsis*, *Amborella* and *Selaginella* (Figure S2, Figure 4) and microalgae [28]. This conservation makes highly probable the involvement of the *P. pinaster* kinases with these signaling elements. SnRK1-2 related bZIP transcription factors are involved in plant abiotic and energetic stress responses in different plant species [41,42]. The overexpression of the *Arabidopsis* bZIP ABF2 confers tolerance to multiple abiotic stresses [43]. Interestingly, the pine PpiSnRK2 could have conserved connections to ethylene signaling and protein glycosylation modulation not observed into the *Arabidopsis* family.

The prediction of the PpiSnRK interactions using species more ancient than *P. pinaster* and *Arabidopsis* (*Amborella, Selaginella* and microalgae) have also allowed the identification of possible SnRK2 interactions to ethylene signaling, protein glycosylation and alternative central regulators as a PP2C-like protein. Despite these connections were not conserved in the *Arabidopsis* network, they would have been inherited by the *P. pinaster* sequences. To overcome pine experimental limitations, model species as *Arabidopsis*, but specially *Selaginella* and *Amborella*—with simpler but close SnRK families—would contribute to the characterization of PpiSnRKs. The knowledge of the specific role of these kinases within the gymnosperm stress response and the identification of their related elements would provide promising targets to drive new selection and breeding strategies. Between these targets would be the PpiSnRKs themselves, orthologs to the conserved bZIP transcription factors and their modulated genes but also novel elements, which are absent from the characterized angiosperms SnRK families. These elements would be interesting candidates for screening programs aimed at the identification of *P. pinaster* genotypes carrying specific sequence changes linked to higher endurance to environmental stress. Previous works based on the characterization of the genotype and secondary metabolism profile among Mediterranean *P. pinaster* populations adapted to different environmental conditions found differences related to the individuals tolerance to stress [5,6]. The candidates found in this work are also an interesting base to identify novel stress associated variations, which could be used as biomarkers of better stress endurance and contribute to breeding/selection programs for the generation of more stress tolerant varieties both in *P. pinaster* and other close gymnosperm species.

5. Conclusions

This work successfully identified and described the SnRK family members of *P. pinaster*, a non-sequenced tree species with a large genome. The success of this approach was based on the search of the orthologs to the well described and characterized *Arabidopsis* SnRKs into the available transcriptome/proteome of *P. pinaster* and other close *Pinus* species. The identification of the gymnosperm SnRKs (PpiSnRK) search was complemented by the prediction of their associations with other stress response-related elements. This prediction required the comparison of the PpiSnRK elements with other known plant SnRK families including *Arabidopsis* and other plant species such as *Amborella* and *Selaginella* with simpler SnRK families. The pine PpiSnRK family showed a high resemblance to those of *Arabidopsis* but also to *Amborella* and *Selaginella*, giving hints about the pine family evolution, structure and function. SnRK2 subfamilies of *Arabidopsis*, *Amborella* and *P. pinaster* shared the same structure divided into ABA sensitive (Groups II, III PpiSnRK2.4 and PpiSnRK2.1, 2.2, 2.3 respectively) and insensitive (group I PpiSnRK2.5, 2.6) elements. This similarity was extended to the SnRK3 subfamily, PpiSnRK3.3 and 3.4 closeness to salt-sensitive *Arabidopsis* SOS2 pointed to the involvement of these PpiSnRK3 into the *P. pinaster* osmotic and/or salt stress response. Moreover, STRING protein interaction networks over related species suggested the conservation of the gymnosperm PpiSnRK2 interaction with known SnRK2 interactors as PP2C, bZIP and SnRK1 while highlighting possible novel interactions. These results supported the use of these model species especially the ones with simpler but close SnRK systems as *Amborella* for the characterization of the gymnosperm SnRKs, overcoming the species experimental difficulties. The knowledge of the specific role of these kinases within the gymnosperm stress response and the identification of their related elements would provide promising targets to drive new selection and breeding strategies. Between these targets would be the PpiSnRKs themselves, orthologs to the conserved bZIP transcription

factors and their modulated genes but also novel elements, which are absent from the characterized angiosperm SnRK families. These elements would be interesting candidates for screening programs aimed at the identification of *P. pinaster* genotypes carrying specific sequence changes linked to higher endurance to environmental stress. These candidates would be used as biomarkers of better stress endurance and contribute to breeding/selection programs for the generation of more stress tolerant varieties both in *P. pinaster* and other close gymnosperm species.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/2/295/s1, Figure S1: M-Coffee alignments of Selaginella and Arabidopsis SnRK2 orthologs, Figure S2: STRING based interaction networks of PpiSnRK orthologs, Table S1: *P. pinaster* PpiSnRK sequence names, clusters, protein and domain identifiers (ID), Table S2: sequences used for the design of the Figure 1 and/or Figure 2 sequence tree.

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