In silico characterization of putative gene homologues involved in somatic embryogenesis suggests that some conifer species may lack LEC2, one of the key regulators of initiation of the process

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Abstract

Background: Somatic embryogenesis (SE) is the process in which somatic embryos develop from somatic tissue in vitro on medium in most cases supplemented with growth regulators. Knowledge of genes involved in regulation of initiation and of development of somatic embryos is crucial for application of SE as an efficient tool to enable genetic improvement across genotypes by clonal propagation.

Results: Current work presents in silico identification of putative homologues of central regulators of SE initiation and development in conifers focusing mainly on key transcription factors (TFs) e.g. BBM, LEC1, LEC1-LIKE, LEC2 and FUSCA3, based on sequence similarity using BLASTP. Protein sequences of well-characterised candidates genes from Arabidopsis thaliana were used to query the databases (Gymno PLAZA, Congenie, GenBank) including whole-genome sequence data from two representative species from the genus Picea (Picea abies) and Pinus (Pinus taeda), for finding putative conifer homologues, using BLASTP. Identification of corresponding conifer proteins was further confirmed by domain search (Conserved Domain Database), alignment (MUSCLE) with respective sequences of Arabidopsis thaliana proteins and phylogenetic analysis (Phylogeny.fr).

Conclusions: This in silico analysis suggests absence of LEC2 in Picea abies and Pinus taeda, the conifer species whose genomes have been sequenced. Based on available sequence data to date, LEC2 was also not detected in the other conifer species included in the study. LEC2 is one of the key TFs associated with initiation and regulation of the process of SE in angiosperms. Potential alternative mechanisms that might be functional in conifers to compensate the lack of LEC2 are discussed.

Keywords: ABSCISIC ACID INSENSITIVE3, BABYBOOM, Conifer, FUSCA3, LEAFY COTYLEDON, PICKLE, SERK, Somatic embryogenesis, WUSCHEL, WOX2

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Background

Somatic embryogenesis (SE) is the process of non-sexual reproduction in which the embryos develop from somatic tissue in vitro on medium in most cases supplemented with growth regulators. Somatic embryos morphologically resemble the zygotic embryos; SE in conifers involves the formation of early-stage somatic embryos, so-called pro-embryogenic masses (PEM), followed by somatic embryo maturation, partial drying with desiccation and germination, giving rise to plants [1]. SE has gained importance not only for its use as a model system in basic studies related to molecular genetics and developmental biology but largely due its application for the large-scale vegetative propagation of plants of uniform quality with selected characters, for commercial purposes [2]. This is of particular interest to the important part of the forest industry based on conifers where the majority of species with large commercial potential are difficult to propagate by traditional cloning methods. In addition, conifers have slow growth, long generation time and very large genome size that makes their genetic improvement difficult and time consuming. SE allows genetic improvements from conifer breeding programs to be captured at an earlier stage and large numbers of high-value plants can be produced [3]. Clonal propagation by SE was successfully demonstrated in coniferous species in the 1980s in *Picea abies* (P. abies [L.] Karst, Norway spruce) [4, 5], then in other genera in the family Pinaceae namely *Abies*, *Larix*, *Picea*, *Pinus* and *Pseudotsuga* [6] and only few species from other conifers belonging to the families Cupressaceae, Taxaceae, Cephalotaxaceae, and Araucariaceae [1]. However, regardless of major technical advances in clonal propagation by SE in conifers, some biological bottlenecks remain. A key step of concern is the limited initiation of SE across genotypes where only a subsection of the seeds can be induced to form a culture of somatic embryos. Furthermore, there are losses in each step during the subsequent development from PEMs to plant lowering the yields. There is only limited information available on the regulation of the SE processes in conifers. Therefore, the identification of key proteins controlling SE with reference to their structural domains deserves primary attention from the conifer perspective. Most investigations in conifers have been focused on the domain characterisation of the WUSCHEL (WUS) and WUS-related homeobox (WOX) protein family [7, 8]. Although expression profiles of some genes associated with SE initiation in conifers have been reported [9], the genetic and molecular interactions in the regulatory network associated with SE development has not been investigated in these species. In addition, there is no information available in conifers regarding the genes involved in suppression of SE (e.g. PICKLE). The motivation for the present study is therefore to summarize information on conifer homologues for the most relevant key regulatory genes involved in SE in model species with the aim to provide a foundation for further detailed studies into functional regulation of the SE process in conifers.

The current work presents in silico identification of putative homologues in conifers to the key regulators of SE based on sequence similarity using BLASTP. These key regulators of SE have been previously identified in *Arabidopsis thaliana* (*A. thaliana*). The analysis includes the identification of putative functional domains of the respective genes, again based on sequence similarity. In addition, relevant information available in the literature with reference to genes associated with SE in conifers has also been reviewed. The analysis focuses mainly on the transcription factors that are demonstrated to be directly involved in the initiation of the SE in the model plants, primarily *A. thaliana*. A few other genes that are known to play significant role during the SE process were also included in the analysis, e.g. SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) which is associated with the initiation of SE and genes like PICKLE (PKL) that are involved in the suppression of SE.

Although PEMs is generally initiated from the immature zygotic embryos in conifers [10], recent studies have also reported SE initiation from the primordial shoot explants and matured embryos of SE plants in *P. abies* [11] and *Picea glauca* (*P. glauca*, White spruce) [12]. Protocols are well established for the induction of SE in various angiosperm plants and gymnosperms including coniferous tree species, yet the information on underlying genetic regulatory mechanism is largely missing. SE in conifers can in most cases be induced by treating the primary explants with plant growth regulators e.g. auxin (2,4-dichlorophenoxyacetic acid) and cytokinin (N6-benzyladenine), or also by wounding or other stress factors e.g. temperature, heavy metal ions, starvation or osmotic stress [13]. Molecular mechanisms governing the regeneration in the explants of coniferous forest tree species with a focus on interaction between auxin and stress conditions have been reviewed [14]. Ectopic and/ or over expression of the key transcription factors involved in the development of SE might also give rise to somatic embryos (discussed in the later part of “Background”).

Genes involved in SE initiation

The core of understanding the SE process lies in the recognition of signals that change the genetic program of somatic tissue to induce the formation of a somatic embryo. This process involves the regulation of gene expression in the somatic tissue that form a somatic
embryo and also in its surrounding tissue. The role of the genes involved in the process of initiation and the regulation of development of the somatic embryos is well characterised in model plants like A. thaliana. The key transcription factors (TFs) which regulate this process include BABYBOOM (BBM), EMBRYOMAKER (EMK), LEAFY COTYLEDON (LEC1, LEC2), LEC1-LIKE (LIL), ABSCISIC ACID INSENSITIVE 3 (ABI3) or VIVI PAROUS (VP1), FUSCA3 (FUS3), WUSCHEL (WUS) and the WUSCHEL-related homeobox (WOX) 2 [15, 16]. These TFs share a complex association with auxin signalling pathways involving a number of gene regulatory networks where various crosstalk and feedback loops play a major role [15, 16]. Seed maturation is synchronised by the complex LAF1 regulatory network, which includes LEC1 and LIL of the NF-YB gene family, and the ABI3/VP1, FUS3 and LEC2 containing the B3 DNA-binding domain and belonging to the B3-AFL gene family. This network positively controls genes involved in embryonic development and maturation and represses those required for the transition from embryonic to vegetative development, suppressing premature germination [17].

LEC1 (LEC1, LEC2, LEC1-LIKE) are among the key regulators that promote the initiation of SE and are involved in the process of early embryo development and maturation [18]. LECs induce formation of somatic embryos when expressed ectopically [19]. Ectopic expression of LIL marked the embryogenic competence in epiphyllous plants [20], while ectopic over-expression of LEC1 [21] and LEC2 [22] was found to be associated with formation of somatic embryos in A. thaliana. By contrast, in conifers, the over-expression of LEC1 homolog gene did not induce ectopic somatic embryo formation in P. glauca but abundance of LECI transcripts was detected in PEMs but not in (non-embryogenic) callus; however in Pinus contorta (P. contorta, Lodgepole pine) [23] and Pinus strobus (P. strobus, White pine) [12], callus also showed expression of the LEC1 homolog. A conifer LEC1-type gene (PaHAP3A) that is active during embryo development in P. abies, did not stimulate embryonic features in vegetative tissues; however, expression of PaHAP3A was observed in embryos and lateral root primordia [36, 37]. BBM acts upstream of other major TFs involved in plant embryo identity as it triggers the LEC1-ABI3-FUS3-LEC2 network to induce SE [38]. Ectopic expression of BBM induces SE in A. thaliana [39]. With reference to conifers, BBM studies have been confined to larch species and P. glauca. Increased expression of BBM was identified during later developmental stages of embryo development in Larix decidua (L. decidua, European larch) [40]. In P. glauca, BBM was observed to be involved in the initiation of SE and was found to be expressed specifically in the early stages of embryo development [12]. BBM along with LEC were proposed to be potential molecular markers for embryogenicity by these investigations. Apart from its involvement in the process of SE in conifers, BBM expression was proposed as a molecular marker for root primordia in hybrid larch (Larix kaempferi × Larix olgensis); BBM showed root-specific expression compared to the gene expression levels in the stem, stem tip and leaf, which indicated that BBM plays a vital role in regulating the development and growth of root during adventitious
rooting in larch [41]. Yet another study concluded the role of BBM (LkBBM1 and LkBBM2) in the regulation of adventitious root development in the same larch hybrid [42].

EMK or AIL5 codes for members of the AP2/ethylene-responsive element binding protein (AP2/EREBP) superfamily having the AP2 DNA-binding domain. EMK is involved in germination and seedling growth, and is essential for the developmental transition between the embryogenic and vegetative phases; over-expression of EMK resulted in the formation of somatic embryos on cotyledons in A. thaliana [43]. Early embryo development is associated with cleavage polyembryony in Pinus species but not in Picea, a process where the proembryo undergoes a cleavage process giving rise to multiple embryos; only one of these embryos develops to a dominant embryo that matures to a cotyledonary embryo, while the other embryos (subordinate embryos) are degraded [44, 45]. Genome-wide transcript expression profiling of early stages of zygotic embryo development in P. sylvestris showed transcript abundance of AIL5 (PsAIL5) along with low expression of VP1 (PsVP1) in subordinate embryos, while PsAIL5 was down-regulated along with up-regulation of PsVP1 in the dominant embryo. This indicated that the transition from the morphogenic phase to the maturation phase was not completed in the subordinate embryos [34].

The WOX family of TFs is comprised of multiple members, of which WUS and WOX2 are associated with the initiation of SE. WUS promotes embryonic identity and vegetative-to-embryonic transition; ectopic WUS expression induces SE in A. thaliana [46]. In P. glauca, PEMs transformed with A. thaliana WUS produced severe phenotypes by disrupting the development of somatic embryos on the maturation medium and inhibiting germination; however WUS did not induce ectopic somatic embryogenesis even in the presence of plant growth regulators [47]. One of the early events in angiosperm embryogenesis is the asymmetric cell division that results in formation of an apical cell which forms the majority of the embryo, and a basal cell which forms the suspensor. WOX2 becomes confined to the apical cell, thus marking the apical descendants of the zygote in A. thaliana involved in its further development [48]. In conifers, there is no corresponding early asymmetric cell division. However, the embryonic region of the early stage conifer embryos constitutes the corresponding tissue responsible for further development of the embryo. High expression of WOX2 is associated with the early growth stages of somatic embryo in P. glauca [12], P. abies [8], P. contorta [23] and P. pinaster [26] and during late embryogeny in P. abies [49]. WOX2 shows evolutionary conserved function related to protoderm formation early during embryo development among seed plants; in addition, it also plays an unique role in suspensor expansion in gymnosperms [49]. Upregulation of a WOX gene was observed during the early to late stages of SE in A. angustifolia [27]. WOX2 expression was much lower at the later embryonal stages in P. abies and it was not detected in non-embryogenic cell culture, therefore it can be used as a marker for embryogenic potential [8, 50]. WOX2 transcripts were found not only in the early to late embryo developmental stages but also in the vegetative tissues of seedlings and mature/older trees in P. abies [8, 51] and P. contorta [23]. Interestingly, WOX2 was found to be expressed in all developmental stages of somatic embryos in P. sylvestris where polyembryony exists, but significantly higher levels of WOX2 expression was detected in subordinate embryos, which might be related to the blocked development of the subordinate embryos [34]. In Cunninghamia lanceolata (C. lanceolata, Chinese fir), however, the WOX2 expression was not associated with the development of the embryos, instead WOX13 transcripts showed high correlation with the transition of PEMs to proembryos [52].

SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINA SE (SERK) belongs to the leucine-rich repeat receptor-like family of kinases (LRR-RLKs) that are involved in multiple processes in plant development. SERK contributes significantly to the process plant embryogenesis and is also found to be involved in diverse plant processes related to cell differentiation, growth and development, and plays important role in plant defense and plant responses to environmental cues [53]. Five SERK genes (SERK1–5) are identified in A. thaliana, where SERK1 forms a component of the embryogenesis signalling pathway [54]. Overexpression of SERK1 enhanced embryogenic competence in tissue cultures of A. thaliana [54]. Expression of SERK1-like was associated with initiation and early SE in P. glauca, as its expression was detected to be higher in the PEMs than in callus, however it was lower in the PEMs than in young shoot buds [12]. A putative homolog of SERK1 gene was found to be expressed in P. sylvestris specifically at the very early stage of embryo development [34]. In A. angustifolia, SERK1 transcripts initially accumulated in the groups of cells at the periphery of the PEMs and were then restricted to the developing embryo [55]. SERK1–3 and SERK1–4 in C. lanceolata share a high similarity with A. thaliana SERK1, and are predominantly expressed in PEMs indicating that both have functions during SE [52].

Genes involved in suppression of SE

PICKLE (PKL) codes for a chromatin re-modeling factor that belongs to the chromodomain-helicase-DNA-binding (CHD) subfamily II. CHD complexes regulate the assembly and organization of mature nucleosomes along
the DNA. CHD proteins are members of ATP-dependent chromatin remodeling complexes that are characterized by presence of the chromo (chromatin organization modifier) domains, SNF2-related helicase/ATPase domain and a DNA-binding domain [56]. PKL is necessary to repress expression of embryonic traits during germination and it regulates the transition from embryonic to vegetative development in A. thaliana [57]. In particular, PKL is necessary for repression of LEC1, a transcription factor, which is one of the key regulators that initiates embryo development [58]. However, there is a lack of information on the function of PKL in conifers.

VP1/ABI3-LIKE (VAL) proteins belong to the plant specific B3 TF superfamily the members of which contains the conserved B3 DNA-binding domain [17]. VALs in A. thaliana contain PHD-L (plant homeodomain-like), Zf (Zinc finger), B3, CW-Zf (named CW for its conserved cysteine and tryptophan residues) and EAR (ethylene response factor [ERF]- associated repression) domains [59]. The B3 domain of VAL mediates the repression of genes of the LAFL network; B3 domain of VAL1 and VAL2 is more similar when compared to VAL3 [60] and all key residues involved in direct DNA contacts are conserved among VAL1 and VAL2 [61]. The PHD and CW-Zf domains are the histone modification readers which are involved in recognition, and the EAR motifs mediate the transcriptional repression [62]. In A. thaliana, VAL1 and VAL2 have been reported as suppressors of somatic embryogenesis [63]; nevertheless, the VAL genes function as suppressors of the LAFL genes during germination, but not during seed development [64]. Knock-down mutations in genes encoding the VAL proteins led to increased expression of LEC genes that resulted in the formation of ectopic somatic embryos on seedlings [65]. Similarly to PKL, the functional mechanism of the action of VAL genes has not been investigated in conifers.

**Results and discussion**

Homologues for all the candidate genes considered as involved in the initiation of SE were detected in the conifer species included in the analysis except one of the key regulators - LEC2. This in silico analysis suggests absence of LEC2 in P. abies and Pinus taeda (P. taeda, Loblolly pine), the conifer species whose genome has been sequenced. Based on available sequence data to date, LEC2 was not detected in the other conifer species included in the study. The details regarding the conifer homologues such as sequence ID, length of the protein etc., are included in the supplementary information (Additional file 1.xlsx). Full-length homologues of the candidate genes were detected in most conifer species with few exceptions; however, our results include all the partial homologues as well, as this aspect is expected to improve with technological advances in the future through availability of elaborate and accurate data e.g. longer reads with PacBio sequencing. In few instances, more than one homologous sequence was detected for a specific candidate gene in a particular conifer species e.g. two BBM gene loci were detected in P. abies and P. taeda. These loci considerably differed in their protein sequences, which can be inferred from the alignment results (Additional file 2.pdf). This phenomenon is also observed with other genes and tree species, e.g. Populus trichocarpa (Torr. & Gray) has one PHYA locus and two PHYB loci, which were designated as PHYB1 and PHYB2 [66]. All homologues from conifer species for a specific candidate gene were aligned along with the corresponding gene from A. thaliana and the characteristic motifs/domains of the respective genes in the conifer homologues are highlighted with different colours and named accordingly with the specific domain names based on the scientific convention as referred from the literature (Additional file 2.pdf - Additional file 11.pdf).

**Conifer homologues of genes involved in SE initiation**

**Homologue of LEC2 was not found in conifer species included in the analysis**

In the current work, LEC2, which is a TF that plays a key role in the initiation and regulation of SE, was found to be absent from the genomes of the P. abies and P. taeda. LEC2 was not detected in the other conifer species included in the study, based on available sequence data in those conifers to date. This observation is strongly supported by the fact that the searches were performed on full genomes of two conifer species involved in this analysis, one each from the genus Picea (P. abies) and Pinus (P. taeda). The phylogenetic tree constructed with conifer homologues of the B3 domain containing TFs (FUS3 and VP1/ABI3, as LEC2 is absent in conifers) and the A. thaliana LEC2, indicates that conifer FUS3 and VP1/ABI3 form separate clusters and the A. thaliana LEC2 forms a distinct clade (Fig. 1). In addition, all the LEC-like conifer homologues showed better alignment with A. thaliana LEC1/LEC1-LIKE than A. thaliana LEC2 (Additional file 3.pdf). This suggests that LEC2 may be absent in conifers, at least in the two conifer species whose whole-genome sequence data is available (P. abies and P. taeda). Furthermore, several transcriptomic investigations related to SE development in conifer species have been conducted but none of them reported the expression of LEC2 [34, 67–69], whereas LEC2 expression is commonly reported in transcriptome analyses in model systems e.g. A. thaliana [70, 71]. Likewise, an earlier study reported that ABI3 homologues were found in all land plant genomes, but the FUS3 homologues were present only in seed plants,
while the LEC2-like sequences were detected only in dicot genomes [72]. Phylogenetic and gene structure analyses of AFL genes (ABI3/VP1, FUS3 and LEC2) in land plant species revealed loss of LEC2 type genes in monocots [17]. This further supports our hypothesis that LEC2 may be broadly absent in conifers.

From the context of loss of genes during evolution, eukaryotic plastid genome has lost many genes during the early events of endosymbiosis; some of these genes were lost totally, while others were found to be relocated and got functionally integrated to the host nuclear genomes during plant evolution [73]. In conifers, loss of ndh genes from several species is evident from plastid genome sequencing projects [74] but later, the presence of non-functional plastid ndh gene fragments was confirmed in the nuclear genome of P. abies [75]. Likewise, there is a specialization of the photosynthetic apparatus in Pinaceae; comparative analysis of the gene families reported gains and losses of genetic networks associated with photosynthesis in Pseudotsuga menziesii (P. menziesii, Douglas-fir) from family Pinaceae [76]. The current analysis suggests loss of LEC2 gene from P. abies and P. taeda, and also from other conifer species included in the study, based on the available sequence information. Embryo development in gymnosperms including conifers is very different from angiosperms in several aspects. For example, the endosperm of gymnosperm is haploid as there is no double fertilization. The conifers possess multiple cotyledons which is a distinctive phenotypic character compared to the monocots and dicots. Few such mechanisms/phenomenon in gymnosperms which are different from the angiosperms, could explain the lack of a master embryogenesis regulator such as LEC2 gene from the conifers.

**LEAFY COTYLEDON1 (LEC1) and LEC1-LIKE**

BLASTP with A. thaliana LEC1 and LEC1-LIKE, resulted in finding the conifer genes characterised as the LEC-like CCAAT-box binding factor HAP3 or the LEC1-type HAP3 subunit coding protein. Congenie displayed A. thaliana LEC1-LIKE as the best match for the conifer homologues detected. The phylogenetic tree constructed with conifer homologues of the LEC sequences precisely indicates that all the LEC/LEC-LIKE conifer homologues either cluster together with A. thaliana LEC1 or A. thaliana LEC1-LIKE sequences (Fig. 2). Here A. thaliana LEC2 is an outgroup which forms a separate clade. All the LEC-like conifer homologues showed alignment with A. thaliana LEC1 and LEC1-LIKE sequences (Additional file 3.pdf). The Asp (D) residue is
critical for the LEC function [77, 78] was found to be conserved in conifers. In addition, the residues unique to LEC1 and LEC1-LIKE HAP3 subunits in the B-domain were found to be conserved in conifers (Figure S57, Additional file 3.pdf); these residues were absent from the B-domain of other HAP3 proteins [79, 80].

**FUSCA3 (FUS3)**
The intact B3 domain is essential for the regulation of seed maturation by FUS3 [81]. Similar to the angiosperms [72, 82], the B domain of FUS3 was more conserved among the conifer species as compared to the N-terminal domain and transcription-activating domain (Figure S16, Additional file 4.pdf). The transcription-activating domain contains conserved FUS3-specific fragments in dicots and in monocots respectively [72]. Likewise, the transcription-activating domain of the FUS3 sequences was found to contain conifer-specific fragments as the transcription-activating domain shows good alignment within the conifer species included in the study but not with *A. thaliana*.

**Viviparous 1 (VP1)**
The VP1 protein contains four domains – A1, B1, B2 and the B3 [35]; the VP1 gene with all the four domains were detected for all the conifer species included in this study. Three homologues of VP1 were detected in *P. abies* but only one sequence (AAG22585.1) showed all four domains (Figure S19, Additional file 5.pdf). One sequence from *P. abies* contained the A1 and the B1 domains (PAB00050494), while the other contained B2 and B3 domains (PAB00050493). We propose that these two sequences may be parts of the same gene but are indicated as separate genes possibly due to annotation and/or sequencing issues. The putative nuclear localization signal (RKNR) of the B2 domain [35] was found to be conserved in all conifer species that showed presence of the B2 domain. The B3 DNA-binding domain of the VP1 genes is well conserved as reported earlier [17] among all conifer homologues and also shows high similarity with *A. thaliana* (Figure S19, Additional file 5.pdf).

**BABYBOOM (BBM)**
BBM is similar to AINTEGUMENTA (ANT), but BBM possesses the characteristic conserved BBM-1 motif (GLSMIKTW); ANT lacks the BBM-1 or the BBM-1 like motif but contains SLSMSPGS motif [83] in *A. thaliana*. The significance of BBM-1 motif was demonstrated in *A. thaliana* where the plants overexpressing BBM gene with a mutated BBM-1 domain failed to produce somatic embryos on cotyledons as compared to the plants bearing the complete CDS of the BBM transgene [84]. Gene structure analysis of *LkBBM1* and *LkBBM2* in hybrid larch revealed that *LkBBM2* protein contained two AP2 DNA binding domains and a BBM specific motif as the *LkBBM1*, but lacked the euANT5 motif common to AP2 family members [42]. However, *LkBBM1* and *LkBBM2* showed similar behaviour with reference to regulation of adventitious root development. These
findings provide concrete evidence regarding the importance of BBM specific motif. BBM proteins of various plant species e.g. A. thaliana (NM_121749, GenBank), Brassica napus (BBM1: AAM33802, BBM2: AAM33801), poplar (BBM1: XM_002316143, BBM2: XM_002311223, GenBank), hybrid larch (BBM1: AHH34920, BBM2: QEL52760, GenBank) and L. decidua contain the GLSMIKTW motif (AEF56566, GenBank). However, variations of the BBM specific motif occur in the maize (Zea mays) and rice (Oryza sativa) proteins. Zea mays BBM contains the ELSMIKTW motif (NP_001147535, GenBank). In rice, three additional genes, Os02g0614300 (OsBBM2), Os01g0899800 (OsBBM3) and Os04g0504500 (OsBBM4) were referred to be homologous to Oryza sativa BABY-BOOM LIKE 1 (Os-BBML1, Os11t0295900) [85] (http://rapdb.dna.affrc.go.jp/). Os-BBML1 and the homologues contain the BBM-1 like motif; Os-BBML1 and OsBBM3 possess the GLSMIKNW motif and, OsBBM2 and OsBBM4 contain the ELSDFKTW motif. OsBBM2 and OsBBM3 function redundantly with OsBBML1 [85].

Fern is a non-seed plant where the BBM gene is absent; it has the ANT gene which lacks the BBM-1 or BBM-1 like motif but possesses the SLSMITGS motif at the same particular location similarly to the A. thaliana ANT (AT4G37750) protein. This ANT gene in fern functionally mimics the BBM gene promoting apogamy [86]; the expression pattern of fern ANT is similar to that of the A. thaliana BBM during early stages of embryo development [39, 86].

The BBM-1 motif and a BBM-1 like motif were detected in the current analysis in conifer proteins – the GLSMIKTW (BBM-1 motif) was found in P. abies, P. taeda and P. sylvestris, and the ELSDFKTW (BBM-1 like) motif was found in Thuja koraiensis (T. koraiensis) (Figure S21, Additional file 2.pdf). The phylogenetic tree constructed with the sequences of conifer homologues of BBM (Fig. 3) shows that the sequences from P. menziesii (PME00019482), P. abies (PAB00065438), P. pinaster (PPI00013750) and P. thunbergii (BAD16602.1) are closer to A. thaliana ANT. BAD16602.1 and PAB00065438 (MA_98095g0010) are characterised as AINTEGUMENTA-like in the respective databases from where the sequences were obtained. Likewise, PPI00013750 is predicted as AP2-like ethylene-responsive transcription factor ANT in Gymno PLAZA,
whereas there is no annotation information available for PME00019482. The BBM-1 motif was not detected in these four sequences; instead they show presence of the GLSALKTW motif. The GLSALKTW motif has higher similarity to the BBM-1 motif (GLSMIKTW) than the motif found in the ANT gene (SLSMSPGS). The requirement of the BBM-1 motif for the proper functioning of the BBM is demonstrated earlier [84]. We propose that the gene homologues found in the conifers, which show homology with the ANT or are characterised as ANT-like genes in either Congenie or Gymno PLAZA but contain the BBM-1 like motif (GLSALKTW), may be the potential BBM-like genes. However, further analysis is required to confirm the functional conservation of these proteins in conifers and angiosperms.

**EMBRYOMAKER (EMK)**
The AP2 subfamily members that are involved in stress responses contain a single copy of the AP2 domain whereas two copies of the AP2 domains are present in the members which play a role in plant development [87]. EMK contains two AP2 domains [43]. In the current study, five conifer sequences were detected with the BLAST searches; three (P. abies, P. sylvestris, P. pinaster) showed two AP2 domains, which may be the precise putative homologues of the EMK genes, while two sequences were found with only one AP2 domain (P. taeda, P. menziesii). The two AP2 domains appear to be conserved among all the sequences in the alignment (Figure S6, Additional file 6.pdf).

**WUS and WUSCHEL-related homeobox (WOX) 2**
WUS homologue was reported by previous investigations related to the analysis of the WOX gene family in P. abies [51] and also in P. pinaster [7]. Although WUS and WOX5 have similar domains (Homeodomain [HD], WUS-box [TL-X-L-F-P] and EAR domain [L-X-L-X-L]), WUS has an extra Y residue in the homeobox domain which is conserved in several plant species [48]. This conserved extra Y residue was earlier reported to be found in the HD of WUS in conifer species e.g. P. abies [51] and also in P. pinaster [7] (Figure S6, Additional file 7.pdf). Only one new WUS homologue was detected in the current analysis in Gymno PLAZA in P. taeda, which also possessed the extra Y residue (PTA00030527). This particular sequence is annotated as WOX4 in the Gymno PLAZA, but since it has the highly conserved extra Y residue in HD, we propose that this is actually the WUS gene. WOX2 contains the HD and WUS-box [48], which was found to be conserved in the conifer species included in this work similar to the earlier studies in conifers [7, 51] (Figure S17, Additional file 8.pdf).

**Somatic embryogenesis receptor kinases (SERK)**
Most of the conifer sequences retrieved with the BLASTP searches with A. thaliana SERK1 were categorised as SERK1 by the respective databases, however a few sequences were referred to as SERK1-like or SERK2 (Additional file 9.pdf). We have included all these sequences in our analysis because SERK1 and SERK2 share 90% identity [88] and these two genes function redundantly while playing a major role in somatic and reproductive cell differentiation as reported during early anther development in A. thaliana [89]. The different domains of SERK1-like or SERK2 were found to be well conserved in all the conifer homologues (Figure S31, Additional file 9.pdf). The SERK1-like or SERK2 conifer homologues contained signal peptide domain, Leucine zipper domain with four conserved Leucine residues, five Leucine rich repeats, the Serine–Proline–Proline (SPP) domain with conserved SPP motifs, the transmembrane domain, the 11 subdomains of the protein kinase domain and the C-teminal domain [90]. Two pairs of cysteine residues were present in the Leucine zipper and SPP domain of the conifer homologues respectively, which were reported to be conserved [91]. The Arginine and Aspartate residues of the subdomain VI of the protein kinase domain, were found to be conserved in all the conifer homologues that contained this domain [90].

**Conifer homologues of genes involved in SE suppression**

**Pickle (PKL)**
PKL acts as a repressor not only for the expression of embryonic traits but also represses the seedling de-etiolation pathway; PKL acts additively with SUPPRESSOR OF PHYTOCHROME A1 (SPA1) to repress seedling de-etiolation and inhibits the protein and transcript levels of ELONGATED HYOCOTYL 5 (HY5) which is one of the important transcription factors that positively regulates the process of photomorphogenesis [92]. PKL physically interacts with HY5 and also with HY5-HOMOLOG (HYH), the close homolog of HY5 to regulate the hypocotyl cell elongation in A. thaliana and interestingly, the ATPase domain of PKL is essential and sufficient for the interaction with both HY5 and HYH [93]. However, a point mutation (Lysine to Alanine) at the position Lys-304 in PKL terminates this interaction [93]. Lys-304 in A. thaliana PKL, is an evolutionarily conserved amino acid that is predicted to bind to ATP within the ATPase domain of PKL. This amino acid was found to be conserved in all the PKL sequences of the conifer species where the ATPase domain was detected, which includes P. abies, P. taeda, P. sylvestris, P. pinaster and P. menziesii (Figure S19, Additional file 10.pdf). Only two PKL sequences, one from P. menziesii and one from P. taeda were found to possess all the known domains of PKL. The detection of partial PKL sequences in
the other conifers maybe because of either lack of data availability due to sequencing quality and/or poor annotation. Yet, it could also be argued that PKL in conifers with only some specific domains acts in a different fashion from what is known in the more advanced angiosperm species, as conifers are known to possess certain specialized pathways compared to the angiosperms e.g. specialization of photosynthetic apparatus in P. menziesii [76]; however, further detailed molecular studies are required to confirm this.

VP1/ABI3-like (VAL)

BLASTP to GenBank with A. thaliana VAL1/VAL2/VAL3 did not give significant matches in Pinidae, while Gymno PLAZA resulted in similar hits with A. thaliana VAL1/VAL2/VAL3 in all the conifer species included in this analysis (with the available sequence data), except in case of P. menziesii where BLASTP searches with VAL1/VAL2 resulted in similar hits but searches with VAL3 gave different matches. There were no significant matches found for A. thaliana VAL1/VAL2/VAL3 in Picea sitchensis (Sitka spruce, P. sitchensis). A. thaliana VAL1 shares 47% identity with A. thaliana VAL2 and 34% identity with A. thaliana VAL3, while A. thaliana VAL2 shares 44% identity with A. thaliana VAL3 as observed from the BLASTP (Additional file 11.pdf). Since similar hits were detected in all the conifer species (included in the analysis), we considered all the sequences together to make the alignments and marked the different domains of the conifer VAL homologues, which were found to be conserved within the conifer species included in the analysis (Figure S67, Additional file 11.pdf). The B3 domain, in particular was found to be highly conserved.

Molecular regulation of genes involved in the initiation of somatic embryogenesis

A schematic model for the mechanism of regulation of initiation of somatic embryogenesis in plants with reference to the key genes involved in the process is summarised in Fig. 4 [13, 16, 18, 94, 95]. Homologues for all the candidate initiation genes except for LEC2 were detected in the conifers. The knowledge in conifers with reference to the initiation of SE is limited to the information regarding the expression patterns of the genes involved and there is lack of evidence for regulation of the process through a gene network. We propose a putative alternative mechanism of the molecular regulation of the process of SE initiation, which may be functional in conifers in absence of LEC2 (Fig. 4) assuming that the...
overall functions of the other genes involved are conserved in conifers. LEC2 is one of the central players in the process of seed and embryo development in plants [18, 19, 22]. In A. thaliana, although LEC2 regulates SE through stimulation of auxin synthesis [96], one of the major roles of LEC2 is to upregulate FUS3 and ABI3; however, ABI3 and FUS3 positively regulate themselves and each other to achieve a uniform expression in the embryo through the feedback loops [97]. LEC1 has also been shown to positively regulate ABI3 and FUS3 expression [97, 98]. Although the expression levels of ABI3 and FUS3 were lowered in LEC2 mutants, constitutive expression of ABI3 or FUS3 was able to rescue the LEC2 phenotypes in A. thaliana [97]. Further, ABI3, LEC2, and FUS3 were proposed to work in parallel pathways and also, FUS3 and LEC2 were shown to act in a partially redundant manner [99]. From this context, the action of ABI3/FUS3 or both may compensate the absence of LEC2 in conifers. Similar to LEC2, LEC1 mediates not only the up-regulation of the auxin synthesis [100] but also facilitates effects of auxin to promote embryonic cell identity [101]. Although BBM and LEC2 regulate each other through a feedback loop, LEC1 and BBM also regulate each other in a similar way [95]. Moreover, BBM also stimulates its own expression through a positive feedback loop to control its own activity [102]. With these assumptions, we propose that in conifers, LEC1 (possibility along with LEC1-LIKE) regulates the network in order to make up for the loss of LEC2. To summarise, SE regulation in conifers may include action of ABI3/FUS3 or both to compensate the absence of LEC2, and the conifer LEC1 along with LEC1-LIKE might be capable of performing adequate functions that are carried out by LEC2. However, further molecular analyses are required to confirm such associated alternative pathways in conifers. Furthermore, conifers exhibit characteristic mechanisms with reference to somatic embryo development such as the presence of cleavage polyembryony in Pinus but broadly not in Picea. Analyses of PEs from more or less polyembryogenic species with respect to known transcription factors involved in somatic embryo regulation in model species could offer insights to regulatory processes active during conifer embryo development. The current work presents fundamental information to support applied studies into underlying molecular mechanisms of conifer somatic embryo initiation and development.

Methods
In this article, we have identified the conifer homologues potentially involved in the initiation of SE. Protein sequences of the candidates genes from A. thaliana were used to query the databases for finding the conifer homologues, using standard protein BLAST (BLASTP) (Basic Local Alignment Search Tool). A. thaliana was chosen as the reference species to detect the conifer homologues as this is the most widely used and the most well-documented model plant species. Databases included in the searches were Gymno PLAZA, 1.0 (https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza/) [104], Congenie (http://congenie.org), v1.0 [105, 106] and GenBank (https://www.ncbi.nlm.nih.gov/genbank/) [107]. BLASTP searches in GenBank were executed by selecting the non-redundant protein sequence database along with selection of Subclass Pinidae (taxid:3313) under the organism option for performing conifer specific searches. Congenie and Gymno PLAZA are platforms for plant comparative genomics; these databases perform the homology searches using BLAST and include the information regarding the best homologues (e.g. best A. thaliana homologue) in the results. Congenie is integrated with gene prediction software e.g. AUGUSTUS and EuGene, which identifies a gene and, it provides the gene description based on Blast2GO, the functional characterization of the gene and the best BLAST homologues. Gymno PLAZA provides the structural and functional annotation of a particular gene, the associated gene family data and phylogenetic trees. However, identity of the particular conifer gene was further confirmed with domain search, alignment and phylogenetic analysis. Specific domains of the particular conifer homologue were identified by performing the search with the Conserved Domain Database (CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) [108]. In addition to CDD search, domains of the particular
conifer gene were also confirmed by referring to the gene specific sequence information available from the literature (Table 1). Furthermore, particular conifer protein sequence of a gene was aligned with the protein sequence of the respective A. thaliana gene using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) [114]. MUSCLE was selected for making the alignments as it uses both global and local alignment algorithms as compared to ClustalW, which uses only global alignment that creates more gaps. Only in case of WOX2, Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) [115] was used which resulted into better alignment related to the WUS box. Phylogenetic trees of protein sequences were constructed for further validation, wherever required, using Phylogeny.fr in the ‘one click mode’ using default settings (https://www.phylogeny.fr/) [116]. In brief, the alignment was done with MUSCLE [114], phylogeny was done using PhyML [117] which is based on the maximum-likelihood principle and the phylogenetic tree was prepared using TreeDyn [118].

Congenie and Gymno PLAZA include the whole genome sequence data from the two representative species from genus Picea (P. abies, v1.0) [105] and Pinus (P. taeda, v1.0) [119] from the Pinaceae family. Other conifers species included in the current in silico analysis were P. abies, P. glauca, P. sitchensis, P. taeda, P. sylvestris, P. pinaster, P. contorta, Pinus massoniana (P. massoniana, Chinese red pine), P. menziesii, A. angustifolia, C. lanceolata, Thuja koraiensis (T. koraiensis, Korean arborvitae), L. decidua and Larix gmelinii var. olgensis x Larix kaempferi (Hybrid larch, L. gmelinii). There were no specific criteria applied for the choice of a particular conifer species included in this analysis, the availability of the data was the prime factor; therefore, all the relevant sequences obtained in the BLASTP results were included in the current analysis.

Table 1 Gene-wise references used for detecting the different domains in the respective genes involved in initiation of somatic embryogenesis in conifers

| Genes involved in initiation of somatic embryogenesis | References |
|-------------------------------------------------------|------------|
| BABYBOOM (BBM)                                       | [41, 42, 83, 84, 109] |
| LEAFY COTYLEDON (LEC)                                | [77–80, 110] |
| FUSCA3 (FUS3)                                        | [72, 82] |
| ABSCISIC ACID INSENSITIVE 3 (ABI3) or VIVIPAROUS (VP1) | [35, 111, 112] |
| EMBRYOMAKER (EMK)                                    | [43] |
| WUSCHEL (WUS) and WUSCHEL-related homeobox (WOX) 2   | [7, 8, 48, 51] |
| Somatic embryogenesis receptor kinases (SERK)         | [90, 91, 113] |

| Genes involved in suppression somatic embryogenesis   | References |
|-------------------------------------------------------|------------|
| PICKLE (PKL)                                          | [58, 108]  |
| VP1/ABI3-LIKE (VAL)                                   | [59, 60, 65] |

Abbreviations
AB3: ABSCISIC ACID INSENSITIVE 3; BBM: BABYBOOM; EMK: EMBRYOMAKER; FUS3: FUSCA3; LEC: LEAFY COTYLEDON; PKL: PICKLE; PEM: Pro-embryogenic masses; SE: Somatic embryogenesis; SERK: Somatic embryogenesis receptor kinases; VP1: VIVIPAROUS; VAL: VP1/ABI3-LIKE; WUS: WUSCHEL; WOX: WUSCHEL-related homeobox

Supplementary Information
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Additional file 1. Details of conifer homologues involved in somatic embryogenesis – Table S1-Table S10.
Additional file 2. Alignments of BBM gene.
Additional file 3. Alignments of LEC gene.
Additional file 4. Alignments of FUS3 gene.
Additional file 5. Alignments of VP1 gene.
Additional file 6. Alignments of EMK gene.
Additional file 7. Alignments of WUS gene.
Additional file 8. Alignments of WOX2 gene.
Additional file 9. Alignments of SERK gene.
Additional file 10. Alignments of PKL gene.
Additional file 11. Alignments of VAL gene.

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Authors’ contributions
SSR was involved in conceptualization, methodology, analysis, representation of the results and manuscript writing – original draft preparation and review & editing. UE was involved in conceptualization, manuscript writing – review & editing, resources and funding acquisition. Both authors have read and approved the manuscript.

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