The plant-specific SCL30a SR protein regulates ABA-dependent seed traits and salt stress tolerance during germination

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Short title: SCL30a regulates ABA-dependent seed traits and salt stress tolerance
Abstract

SR (serine/arginine-rich) proteins are conserved RNA-binding proteins best known as key regulators of splicing, which have also been implicated in other steps of gene expression. Despite mounting evidence for their role in plant development and stress responses, the molecular pathways underlying SR protein regulation of these processes remain elusive. Here we show that the plant-specific SCL30a SR protein negatively regulates abscisic acid (ABA) signaling to control important seed traits and salt stress responses during germination in Arabidopsis. The SCL30a gene is upregulated during seed imbibition and germination, and its loss of function results in smaller seeds displaying enhanced dormancy and elevated expression of ABA-responsive genes as well as of genes repressed during the germination process. Moreover, the knockout mutant is hypersensitive to ABA and high salinity, while transgenic plants overexpressing SCL30a exhibit reduced ABA sensitivity and enhanced tolerance to salt stress during seed germination. An ABA biosynthesis inhibitor rescues the mutant’s enhanced sensitivity to stress, and epistatic analyses confirm that this hypersensitivity requires a functional ABA pathway. Finally, seed ABA levels are unchanged by altered SCL30a expression, indicating that the SR protein positively regulates stress tolerance during seed germination by reducing sensitivity to the phytohormone. Our results reveal a new key player in ABA-mediated control of early development and stress response, and underscore the role of plant SR proteins as important regulators of the ABA signaling pathway.
Author Summary

Seed germination is a critical step in plant development determining the transition to aerial growth and exposure to a more challenging environment. As such, seeds have evolved mechanisms that prevent germination under adverse conditions, thereby increasing the chances of plant survival. As a general regulator of plant development and a key mediator of stress responses, the hormone abscisic acid (ABA) promotes a prolonged non-germinating state called dormancy, influences seed size and represses germination under environmental stress.

Here, we show that an RNA-binding protein, SCL30a, controls seed size, dormancy, germination and tolerance to high salinity in the model plant Arabidopsis thaliana. Loss of SCL30a gene function results in smaller and more dormant seeds with reduced ability to germinate in a high-salt environment; by contrast, SCL30a overexpression produces larger seeds that germinate faster under salt stress. Using a large-scale gene expression analysis, we identify the ABA hormonal pathway as a putative target of SCL30a. We then use genetic and pharmacological tools to unequivocally demonstrate that the uncovered biological functions of SCL30a are achieved through modulation of the ABA pathway. Our study reveals a novel regulator of key seed traits and has biotechnological implications for crop improvement under adverse environments.
**Introduction**

Seed germination begins with rehydration (imbibition) and expansion of the embryo by cell elongation, which leads to rupture of the weakened seed coat and emergence of the radicle [1]. During water uptake, a prolonged non-germinating state termed seed dormancy must be relieved before protrusion of the radicle [2]. The completion of seed germination marks a key developmental milestone in the life cycle of higher plants, being essential for the establishment of a viable plant. The germination process is highly regulated by both endogenous and environmental signals that determine the dormancy status of the seed and its aptitude to germinate [3].

The plant hormone abscisic acid (ABA) promotes seed maturation and dormancy while inhibiting seed germination, thus acting as a key regulator of this critical developmental step [4,5]. In fact, mutations that affect components of ABA biosynthesis (e.g., \textit{aba2}) or signaling (e.g., \textit{snrk2.2/3/6}) exhibit reduced seed dormancy and precocious germination [6,7]. ABA has also more recently been implicated in the control the seed’s final size, with ABA production and signaling modulating the expression of \textit{SHB1}, a main regulator of endosperm cellularization during seed development [8].

Apart from regulating key developmental processes such as seed germination, ABA is a known major mediator of osmotic stress responses, also in seeds where it acts as an integrator of different environmental signals to repress germination under unfavorable conditions [5]. While numerous studies have deciphered the genetic components and transcriptional mechanisms underlying seed germination and osmotic stress responses, the involvement of posttranscriptional gene regulation, namely of alternative splicing, is beginning to unfold [9–11].

RNA splicing, which excises introns from the precursor mRNA (pre-mRNA) and joins the flanking exonic sequences to generate mature transcripts, is an essential step in eukaryotic gene...
expression. This process involves the recognition of intronic sequences called splice sites by
the spliceosome, a large molecular complex consisting of five small nuclear ribonucleoproteins
(snRNPs) and numerous spliceosome-associated proteins that assemble at introns in a precise
order [12,13]. The differential recognition of splice sites results in alternative splicing, which
allows a single gene to express multiple mRNA variants and hence greatly contributes to
transcriptome diversification.

SR (serine/arginine-rich) proteins are multi-domain, non-snRNP spliceosomal factors that
regulate pre-mRNA splicing. These RNA-binding proteins use one or two of their N-terminal
RNA recognition motifs (RRMs) to bind to specific cis-acting elements in pre-mRNAs and
enhance or repress splicing [14]. SR proteins recruit core spliceosomal factors to pre-mRNAs
through their C-terminal arginine/serine (RS) domain, which acts a protein-protein interaction
module [15]. The RS domain is also subjected to numerous reversible phosphorylation events
that control SR protein activity and subcellular localization [16,17].

Apart from pre-mRNA splicing, non-canonical functions for SR proteins in pre-and post-
splicing activities have been emerging, highlighting their multifaceted roles as important
coordinators of nuclear and cytoplasmic gene expression machineries [18,19]. In one example,
the mammalian SR protein SRSF2 was shown to mediate the activation of the paused Pol II by
releasing the positive transcription elongation factor b (p-TEFb) from inhibitory 7SK
ribonucleoprotein complexes, thus promoting transcriptional elongation [20]. Furthermore,
changes in SRSF2 levels have been shown to affect the accumulation of Pol II at gene loci [21].

More generally, SR proteins influence gene transcription by directly or indirectly interacting
with the C-terminal domain of RNA Pol II during their assembly as RNA processing factors
[18]. Animal SR proteins have also been shown to influence mRNA export, translation and
decay by interacting with major components of the molecular complexes regulating these
processes [19].
Functional analyses of individual SR and SR-like proteins in plants have identified specific roles for these proteins in stress and ABA responses. The Arabidopsis RS40 and RS41 were found to interact in nuclear speckles with HOS5, a KH-domain RNA-binding protein, and FRY2/CPL1, a major player in the co-transcriptional processing of nascent transcripts, with knockout mutants of these two SR proteins displaying hypersensitivity to ABA during seed germination as well as to the inhibitory effect of salt on root elongation [22]. RSZ22 is a putative dephosphorylation target of the Clade A protein phosphatase 2C HAI1, a major component of ABA and osmotic stress signaling in Arabidopsis [23]. The SR-like SR45a was recently shown to inhibit salt stress tolerance in Arabidopsis by interacting with the RNA cap-binding protein CBP20 and regulating alternative splicing of transcripts involved in the response to high salinity [24]. In addition to salt stress responses [25], the other Arabidopsis SR-like protein, SR45, regulates sugar responses by repressing both ABA signaling and glucose-induced accumulation of the hormone [26,27], with SR45-bound transcripts being markedly enriched in ABA signaling functions [28]. In support of a conserved role for these proteins in splicing regulation, SR45 and SR45a interact with the spliceosomal components U1-70K and U2AF35b [24,29] involved in the recognition of 5’ and 3’ splice sites, respectively.

The Arabidopsis genome encodes 18 SR proteins, 10 of which are orthologs of the human ASF/SF2, 9G8 or SC35, while members of the RS, RS2Z and SCL subfamilies are plant-specific [30]. SCL30a belongs to the latter subfamily, whose four members are similar to SC35 but display a distinctive short N-terminal charged extension rich in arginines, serines, glycines and tyrosines [30]. SCL30a interacts with the RS2Z33 SR protein [31] and acts redundantly with its paralog SCL33 to control alternative splicing of a specific intron in the SCL33 pre-mRNA [32]. A more recent study described pleiotropic developmental phenotypes for a quintuple mutant of the four SCL and the SC35 genes, including serrated leaves, late flowering,
shorter roots and abnormal siliques and phyllotaxy, while the corresponding single mutants did not show obvious phenotypic alterations [33]. Furthermore, all four SCL members (SCL28, SCL30, SCL30a and SCL33) and SC35 localize in nuclear speckles and interact with major components of the early spliceosome machinery U170K and U2AF65a [33], corroborating their function as splicing regulators. Interestingly, these five Arabidopsis SR proteins were also recently reported to interact with the NRPB4 subunit of RNA Pol II, indicating a potential role in the regulation of transcription [33].

Here, we characterized the plant-specific SCL30a gene in Arabidopsis and found that the encoded protein regulates seed size, dormancy and germination. Loss of SCL30a function affects alternative splicing of a limited number of genes, but upregulates expression of many osmotic stress and ABA-responsive genes. In agreement with this, the scl30a-1 loss-of-function mutant displays strong hypersensitivity to ABA and salt stress during seed germination. Conversely, overexpression of SCL30a reduces ABA sensitivity and confers seed tolerance to salt stress during germination. Epistatic and pharmacological analyses demonstrate that SCL30a’s function in seeds and salt stress tolerance depends on ABA synthesis and signaling, demonstrating a key role for this RNA-binding protein in ABA-mediated responses.

**Results**

**SCL30a expression is markedly induced during seed germination**

To initiate the characterization of the Arabidopsis SCL30a gene and investigate its expression pattern, we generated transgenic plants expressing the β-glucuronidase (GUS) reporter gene under the control of the SCL30a endogenous promoter. The SCL30a promoter was active throughout plant development (Fig 1A). We observed GUS staining in vascular tissues and actively dividing cells, such as in the shoot meristem and young leaves (Fig 1A (a)), the primary root tip (Fig 1A (b)) and lateral root primordia (Fig 1A (c)). At the reproductive phase, SCL30a...
appeared to be particularly expressed in the pistil tip, the vasculature tissue of sepals, the stamen filaments and pollen grains (Fig 1A (d)) of developing flowers. In embryonic tissues, the $SCL30a$ promoter was active from the early — globular and heart (Fig 1A (e-g)) — to the late — torpedo and mature embryo (Figure 1A (h-j)) — stages of embryo development. Finally, in imbibed mature seeds, GUS staining was detected in the whole embryo (Fig 1A (k)) as well as strongly in the seed coat (Fig 1A (l)), but is mainly expressed at the radicle tip during germination (Fig 1A (m)).

In parallel, we used RT-PCR to study the development- and tissue-specific expression pattern of $SCL30a$. Consistent with the established promoter:GUS expression profile, $SCL30a$ was expressed both in young seedlings and at later developmental stages, with its mRNA being detected in different aerial tissues, such as leaves, stem, flowers and siliques, but also in roots (Fig 1B). In embryonic tissues, although $SCL30a$ transcripts were undetectable in dry seeds, gene expression was clearly observed at 3 days of seed imbibition at 4 °C and increased sharply during the first hours of germination upon transfer to 22 °C and light (Fig 1B).

Both animal and plant pre-mRNAs encoding splicing components appear to be particularly prone to alternative splicing themselves. This has been shown to lead to a dramatic increase of the transcriptome complexity of the Arabidopsis SR protein family [34,35], prompting us to examine the splicing pattern of the $SCL30a$ gene. Although only one transcript has been annotated (www.arabidopsis.org), cloning and sequencing of the PCR products amplified from the $SCL30a$ cDNA identified three alternative mRNAs (S1 Fig), consistent with the information available in PASTDB (http://pastdb.org.eu), a recently developed transcriptome-wide resource of alternative splicing profiles in Arabidopsis [36]. The shortest and by far most expressed $SCL30a.1$ transcript (Fig 1B and S1B Fig) is predicted to encode the full-length protein, while the other two splice variants encode putative severely truncated proteins (S1A Fig).
Thus, the Arabidopsis SCL30a gene, which produces at least three alternative transcripts, displays ubiquitous expression in vegetative tissues and is induced during seed germination.

**Loss of SCL30a function reduces seed size, enhances seed dormancy and delays germination**

To investigate the biological roles of SCL30a, we isolated a homozygous T-DNA mutant line, SALK_041849, carrying the insertion in the gene’s third exon (S1A Fig). RT-PCR analysis of SCL30a expression in this scl30a-1 mutant using primers annealing upstream of the insertion site revealed transcript levels comparable to the Col-0 wild type, but no expression was detected when primers flanking or annealing downstream of the T-DNA were used (S1B Fig). Consistent with the location of the insertion, no splice variants were detected in the mutant, which only expresses a truncated SCL30a transcript lacking the sequence corresponding to the entire RS domain as well as most of the RRM (S1 Fig). These results indicate that the scl30a-1 allele is a true loss-of-function mutant.

Given that we observed no notable defects in adult plants and the marked induction of the SCL30a gene during seed imbibition and germination (see Fig 1), we focused our phenotypical analysis of the scl30a-1 mutant on embryonic tissues. Notably, mature scl30a-1 seeds displayed a significant reduction in size, with dry and imbibed mutant seeds being 12 % and 14 % smaller, respectively, than seeds of wild-type plants (Fig 2A). Correlating with their smaller size, dry mature scl30a-1 seeds showed reduced weight when compared to the wild type, but no significant changes in their relative moisture, protein or oil content (S1 Table). A more detailed compositional analysis revealed only minor changes in the relative levels of a few unsaturated fatty acids and the trisaccharide raffinose (S1 Table), indicating that loss of SCL30a function does not substantially affect nutrient and water storage in embryonic tissues. Interestingly, the scl30a-1 mutant also exhibited enhanced seed dormancy. After 7 days at 22 °C in darkness, the germination rate of freshly-harvested, non-stratified scl30a-1 seeds was
only about one third of that of wild-type seeds (Fig 2B). The germination rate of stratified
scl30a-1 mutant seeds was slightly lower, exhibiting a significant delay when compared to
wild-type seeds (Fig 2C).

The seed phenotypes of the scl30a-1 mutant prompted us to analyze the expression of the
ABI3 and ABI5 genes, two major transcriptional regulators controlling seed development,
dormancy and germination [37,38]. RT-qPCR analyses of germinating seeds showed that the
expression of ABI5, and to a lesser extent also ABI3, is significantly increased in the scl30a-1
mutant (Fig 2D). In agreement, the expression of Em1, Em6, and LEA4-5, three downstream
targets of the ABI3 and ABI5 transcription factors [39,40], was also upregulated in scl30a-1,
even to a larger extent (Fig 2D).

These findings indicate that the SCL30a SR protein plays an in vivo role in embryonic
tissues, where it affects seed size, dormancy and germination, and controls the expression of
key genes regulating seed development and germination.

The SCL30a protein affects alternative splicing of a small set of genes during seed
germination

To gain insight into the molecular functions of the SCL30a RNA-binding protein, we next
conducted an RNA-sequencing (RNA-seq) experiment to compare the transcriptomes of wild-
type and mutant germinating seeds. We used the Illumina HiSeq 2500 system to sequence
mRNA libraries prepared from Col-0 and scl30a-1 seeds 18 h after stratification and obtained
a minimum of 90 million paired-end clean sequence reads per sample. Given the conserved
role of SR proteins in pre-mRNA splicing, we first analyzed the alternative splicing changes
cau sed by the scl30a-1 mutation.

At the time point sampled, we found only 22 alternative splicing events in 21 genes to be
differentially regulated in scl30a-1 mutant seeds (ΔPSI > |15|): seven intron retention (IR),
three exon skipping (ES) and 12 alternative 3’(Alt3) or alternative 5’ (Alt5) splice site events
Although of low magnitude (ΔPSI < 25 in all cases), the RNA-seq alternative splicing changes were confirmed in the four events selected for validation by RT-PCR, using wild-type and scl30a-1 RNA samples independent from those analyzed by RNA-seq (S2 Fig). Interestingly, all of the seven differentially-regulated IR events showed lower inclusion levels in the scl30a-1 mutant, suggesting that SCL30a negatively regulates splicing of these introns. On the other hand, for the three differential ES events identified, the exons were more included in the wild type, pointing to a role of SCL30a in promoting splice site recognition. Thus, IR and ES results point to a contradictory role of this protein in splicing regulation; however, the number of alternative splicing events retrieved is too low to draw conclusions on the mechanistic function of SCL30a.

### Table 1. Genes displaying alternative splicing changes in germinating scl30a-1 mutant seeds.

Means of percent spliced-in index (PSI) in Col-0 wild-type (WT) and scl30a-1 mutant germinating seeds (n = 3) are presented. Genes are ordered by type of alternative splicing (AS) event — intron retention, (IR, in red), exon skipping (ES, in orange), alternative 5’ splice site (Alt5, in blue) and alternative 3’ splice site (Alt3, in green) — and decreasing absolute ΔPSI values.

| Gene ID     | Gene name | AS event ID     | AS event type | WT PSI | scl30a-1 PSI | ΔPSI |
|-------------|-----------|-----------------|---------------|--------|--------------|------|
| AT3G07420   | SYNC2     | AthINT0106607   | IR            | 54.1   | 35.1         | 19.0 |
| AT2G19910   | RDR3      | AthINT0098183   | IR            | 29.1   | 12.2         | 16.9 |
| AT3G07890   |           | AthINT0022974   | IR            | 26.0   | 9.2          | 16.8 |
| AT1G06500   |           | AthINT0001122   | IR            | 53.1   | 36.6         | 16.5 |
| AT2G46915   |           | AthINT0021222   | IR            | 39.0   | 22.5         | 16.5 |
| AT5G64980   |           | AthINT0051338   | IR            | 37.1   | 20.7         | 16.4 |
| AT5G09690   | MGT7/MRS2-7 | AthINT0086682 | IR            | 27.8   | 12.2         | 15.6 |
| AT3G63445   |           | AthEX0008460    | ES            | 97.3   | 72.4         | 24.9 |
| AT1G45248   |           | AthEX0002068    | ES            | 70.5   | 52.9         | 17.6 |
| AT1G10890   |           | AthEX0000589    | ES            | 42.4   | 25.4         | 17.0 |
| AT1G15410   |           | AthALTD0000759-2/2 | Alt5     | 89.0   | 68.6         | 20.3 |
Of the 21 genes differentially spliced in the *scl30a-1* mutant, only three have been characterized previously: *MRS2-7* encodes a magnesium transporter [41], *POT1a* a DNA-binding protein required for telomere maintenance [42] known to be regulated by alternative splicing [43], and *SGF29a* is a transcriptional co-activator implicated in salt stress responses [44]. Based on the gene annotation at TAIR (www.arabidopsis.org), another four genes appear to be also involved in transcription or different aspects of RNA metabolism, while seven play putative roles in many different processes, including lipid or nitrogen metabolism, glycolysis, cell division and protein deubiquitination. Yet, one third of the genes whose splicing was found to be affected by the SCL30a SR protein (i.e., seven genes) are of unknown function.

The SCL30a protein regulates transcriptional responses related to seed germination and ABA

We next analyzed the gene expression changes caused by loss of function of the *SCL30a* gene. Our RNA-seq analysis revealed 382 genes whose expression was significantly changed by at least two-fold in the *scl30a-1* mutant. Among these, 315 displayed higher transcript levels than the wild type, whereas 67 were downregulated in the *scl30a-1* mutant (S2 Table).

Given the seed and germination phenotypes of the *scl30a-1* mutant (see Fig 2), we then asked whether the genes whose expression was affected by the SCL30a protein were transcriptionally regulated during the seed germination process. To address this question, we
quantified the expression levels of the genes up- and downregulated in the *scl30a-1* mutant using data from an extensive germination time-course RNA-seq experiment performed by Narsai et al. [9] (Fig 3). Remarkably, we found that genes repressed by SCL30a (i.e., upregulated in the mutant) are in general highly expressed in dry seeds and downregulated throughout the germination process (Fig 3A). Conversely, genes whose expression is activated by SCL30a (i.e., downregulated in the mutant) show the opposite trend, being lowly expressed in dry seeds and induced during germination (Fig 3B). This finding coincides with the expression pattern of SCL30a (see Fig 1) as well as with the delay in germination exhibited by the *scl30a-1* mutant (see Fig 2C) and points to this SR protein as an important positive regulator of seed germination.

Importantly, among the genes upregulated in the *scl30a-1* mutant we found many involved in embryo development, seed maturation and dormancy. They include seed storage proteins (e.g., CRUCIFERIN) and genes involved in the accumulation and storage of lipidic compounds in seeds (e.g., oleosins), as well as genes involved in the acquisition of desiccation tolerance, such as many LATE EMBRYOGENESIS ABUNDANT (LEA) genes (S2 Table). In agreement, Gene Ontology (GO) analysis of the *scl30a-1*-upregulated genes showed clear enrichment for categories related to these developmental processes, such as GO:0045735: nutrient reservoir activity, GO:0019915: lipid storage, GO:0009414: response to water deprivation or GO:0009793: embryo development ending in seed dormancy (Fig 4A and S3 Table). Moreover, consistent with the key role played by the ABA hormone in the regulation of seed development, maturation, dormancy and germination, the functional category “GO:0009737: response to abscisic acid” appeared strongly enriched among the *scl30a-1*-upregulated genes. Indeed, the expression of genes encoding main regulators and targets of the ABA signaling pathway — including the ABI5 bZIP transcription factor [45], the seed-specific PP2C AHG1 [46] and the ABA-responsive dehydrin RAB18 [47] — was found to be significantly enhanced
in the mutant (Fig 2D and S3 and S4 Tables). On the other hand, many genes found to be
downregulated in the scl30a-1 mutant were related to microtubule activity and cell wall
remodeling (Fig 4B and S3 Table), two important processes known to be activated during
germination of the seed [1,48].

To gain further insight into the extent of SCL30a control of ABA responses during seed
germination, we compared the differentially expressed genes in the scl30a-1 mutant with a list
of ABA-regulated genes obtained from the reanalysis of a previous microarray experiment
performed in germinating seeds submitted to a transient ABA treatment [49]. Strikingly, 80 %
(252 genes) of the genes upregulated in the scl30a-1 mutant were also induced by ABA in
wild-type germinating seeds (Fig 4C and S5 Table), while 49 % (33 genes) of the genes
downregulated in the scl30a-1 mutant were repressed by ABA (Fig 4D and S6 Table). We then
analyzed the expression levels of the ABA-regulated genes defined based on [49] in our RNA-
seq data. Interestingly, the 1446 genes upregulated by ABA were significantly more highly
expressed in scl30a-1 than in the wild type, while the 1675 ABA-downregulated genes were
downregulated in our mutant (Fig 4C and 4D). Together, these results suggest that an important
component of SCL30a function during seed germination is related to the control of ABA-
mediated transcriptional responses.

SCL30a is a positive regulator of ABA signaling and salt stress tolerance during seed
germination

To further characterize and confirm the functional role of the SCL30a SR protein in seeds, we
generated transgenic Arabidopsis lines expressing the full-length SCL30a.1 transcript under
the control of the 35S promoter in the wild-type Col-0 background. Three independent lines
noticeably overexpressing the SCL30a.1 mRNA, SCL30a-OX1, SCL30a-OX2 and SCL30a-
OX3 (Fig 5A and S3A Fig), were selected for phenotypical characterization. We first assessed
the impact of SCL30a overexpression on the seed traits found to be affected by the scl30a-1
mutation (see Fig 2). In contrast to what was observed for the scl30a-1 mutant, imbibed seeds from the SCL30a-overexpressing plants were significantly (10%) larger than those from wild-type plants (Fig 5B and S3B Fig). Furthermore, stratified SCL30a-overexpressing seeds germinated slightly faster under control conditions than wild-type seeds (Fig 5C).

The differential expression of ABA-related genes observed in the scl30a-1 mutant prompted us to analyze ABA response of the different genotypes during germination. We found that the scl30a-1 mutant displays strong hypersensitivity to the hormone (Fig 5D and S3C Fig), with less than 10% of the mutant seeds germinating under ABA concentrations that allowed 75% germination of the wild type (Fig 5D). In agreement, seeds from the SCL30a overexpression lines were less sensitive to the hormone during seed germination (Fig 5D and S3C Fig). Given the established link between ABA and osmotic stress responses [5], we next examined the effects of loss of function and overexpression of the SCL30a gene on seed germination under salt stress. In line with the effect of exogenously applied ABA, the germination rate of mutant seeds in the presence of 200 mM of NaCl was markedly reduced when compared to those of the wild type, while the SCL30a overexpression lines were hyposensitive to high salinity, germinating twice as well as the wild type under these conditions (Fig 5D and S3C Fig).

The above findings show that the full-length SCL30a SR protein plays an in vivo role in seed development and germination, clearly substantiating the notion that it positively regulates seed size and germination. Moreover, the strikingly opposite phenotypes under ABA and salt stress induced by loss of function and overexpression of SCL30a demonstrate that this Arabidopsis SR protein is a positive regulator of osmotic stress tolerance during germination of the seed.

**SCL30a function in seeds depends on the ABA pathway**

To investigate whether the role of SCL30a in salt stress responses is mediated by ABA, we first performed stress germination assays in the presence of fluridone, an inhibitor of ABA
biosynthesis [50–52]. Consistent with the well-known role of ABA as a key mediator of salt stress responses [5], addition of 1 μM fluridone notably relieved the inhibition imposed by NaCl on the germination of wild-type seeds (Fig 6A). Most importantly, the presence of fluridone rescued the salt stress hypersensitive phenotype of the scl30a-1 mutant, which germinated at rates similar to the wild type in NaCl (Fig 6A). This result indicates that the mutant’s salt stress germination phenotype depends on endogenous ABA production.

To conclusively establish the ABA dependence of SCL30a function, we next turned to epistatic analyses and assessed the genetic interaction between SCL30a and ABA2, encoding a cytosolic short-chain dehydrogenase reductase involved in the conversion of xanthoxin to ABA-aldehyde during ABA biosynthesis [53], or ABI4, which encodes an ERF/AP2-type transcription factor involved in ABA signal transduction [54,55]. To this end, the scl30a-1 mutant was independently crossed with the ABA-deficient aba2-1 [6] and ABA-insensitive abi4-101 [56] mutant alleles to generate the corresponding homozygous double mutants. As seen in the dose-response curves depicted in Fig 6B, seeds from the scl30a-1aba2-1 and scl30a-1abi4-101 double mutants behaved as those of the corresponding single ABA mutants when germinated under high salinity, showing that SCL30a control of this stress response fully relies on functional ABA2 and ABI4 genes.

We then assessed the seed size and dormancy of the different genotypes. Both the aba2-1 and the abi4-101 mutations suppressed the reduced size displayed by scl30a-1 seeds, with the area of scl30a-1aba2-1 imbibed seeds being even significantly larger than those of the wild type, as previously reported for the aba2-1 mutant [8] (Fig 6C). Regarding seed dormancy, the double mutants again showed strikingly similar phenotypes to those induced by single mutations in the ABA2 and ABI4 genes that, in agreement with early reports [6,57], conferred strongly reduced and normal dormancy, respectively (Fig 6D). Therefore, both ABA2 and ABI4
are epistatic to the SCL30a gene, indicating that the seed/germination roles of the encoded SR protein are fully dependent on a functional ABA pathway.

The above findings raised the question of whether changes in SCL30a levels affect ABA biosynthesis or sensing/signaling of the stress hormone. To address this issue, we measured the endogenous ABA content of wild-type, scl30a-1 mutant and SCL30a-overexpressing seeds germinated in control conditions or under high salinity stress. Table 2 shows that Col-0, scl30a-1 and SCL30a-OX seeds responded to the presence of 200 mM NaCl by increasing their ABA content by around two-fold, with no significant differences in ABA levels being observed between the three genotypes either in the absence or presence of salt stress. As expected, the ABA content of the ABA biosynthesis aba2-1 mutant, included as a negative control, was unaltered by high salinity stress (Table 2). These results suggest that SCL30a activity does not influence endogenous ABA levels in seeds, rather affecting sensing and/or signal transduction of the hormone during seed germination.

**Table 2. Effect of loss of function or overexpression of SCL30a on seed ABA levels.**

ABA content (means ± SE, n = 6-8), in ng/g of fresh weight, of Col-0, scl30a-1, SCL30a-OX2 and aba2-1 seeds germinated for 2 days in the absence or presence of 200 mM NaCl. Letters indicate significantly different ABA levels between genotypes among each condition and asterisks significant differences for each genotype between control and salt stress conditions (p < 0.05; Student’s t-test).

| Genotype   | Control     | NaCl        | NaCl/Control |
|------------|-------------|-------------|--------------|
| Col-0      | 31.62 ± 3.07| 59.06 ± 8.40| *1.86 ± 0.40 |
| scl30a-1   | 36.45 ± 7.40| 73.04 ± 16.60| *2.00 ± 0.80 |
| SCL30a-OX2 | 36.54 ± 7.29| 89.00 ± 19.91| *2.44 ± 0.98 |
| aba2-1     | 27.86 ± 6.61| 24.25 ± 4.11*| 0.87 ± 0.35  |
Discussion

The first indication that the Arabidopsis SCL30a SR protein was involved in regulating seed-specific traits came from our gene expression studies, showing high SCL30a induction in the embryo and testa of imbibed seeds as well as during the first stages of germination. Phenotypical characterization of the scl30a-1 loss-of-function mutant then revealed that this gene is required to achieve the final size and adequate dormancy levels of mature Arabidopsis seeds, as well as subsequently during the germination process. Importantly, we also show that SCL30a negatively regulates the response to salt stress as well as ABA signaling during germination of the seed. Accordingly, germinating scl30a-1 mutant seeds display higher expression of ABA-related genes, and overexpression of SCL30a results in a drastic reduction of seed sensitivity to high salinity, corroborating a role for this protein as a positive regulator of abiotic stress tolerance during seed germination.

Although the SCL30a gene displays ubiquitous expression in vegetative tissues, we were unable to identify any evident phenotype at later developmental stages. This is likely due to functional redundancy between members of the SCL subfamily at the adult stage. In fact, previous phenotypic studies of adult Arabidopsis plants from single mutants in individual SCL genes did not report any visible alterations, with only a quintuple mutant of the four SCL members and the SC35 gene (scl28 scl30 scl30a scl33 sc35) exhibiting clear defects in leaf development and flowering [33].

Physiological assays using an ABA biosynthesis inhibitor and epistatic analyses with the ABA-biosynthesis ABA2 [6] and the ABA-signaling ABI4 [57] genes demonstrate that SCL30a regulation of seed traits is fully dependent on an intact ABA pathway. This is consistent with the global transcriptional changes associated with the loss of SCL30a function, showing a clear enrichment of ABA-related functions among the genes upregulated in the scl30a-1 mutant. Moreover, unchanged ABA levels in mutant and overexpressing seeds, together with the
enhanced and reduced sensitivity to exogenously applied ABA caused respectively by loss-of-
function and overexpression of SCL30a, indicate that the encoded SR protein represses signal
transduction of the phytohormone rather than its biosynthesis.

While the central roles of ABA in the induction and maintenance of seed dormancy as well
as in mediating responses to salt stress are well established [5], few studies have addressed the
involvement of this phytohormone in determining seed size. Nonetheless, expression of the
ubiquitin interaction motif-containing DA1 protein, which limits seed size by restricting the
period of cell proliferation in the seed integuments, is induced by ABA and a da1 mutant allele
displays altered ABA sensitivity. However, unlike SCL30a, DA1 function appears to be
independent of the ABI4 gene [58]. ABA has also been reported to regulate final seed size via
the control of endosperm cellularization during seed development, as reflected by the larger
seeds of the aba2 and abi5 mutants [8]. Given the smaller seeds produced by the scl30a-1
mutant and the newly discovered role for SCL30a as a major regulator of ABA transcriptional
responses, it appears more likely that this SR protein regulates endosperm development, and
thereby seed size, by controlling the expression of key ABA components such as the ABI5
gene, which is upregulated in the scl30a-1 mutant.

Seeds challenged with osmotic stress undergo an arrest in germination that is triggered by a
rise in their ABA content [59,60]. Our results indicate that by decreasing sensitivity to this
phytohormone, the SCL30a SR protein enhances salt stress tolerance during seed germination.
The derepression of a subset of ABA-response genes and the germination delay associated with
the loss of SCL30a function in the absence of stress suggest that the SR protein is already able
to repress ABA signaling under optimal growth conditions. Therefore, it is possible that the
hypersensitive phenotype of the scl30a-1 mutant is a consequence of an already active ABA
signaling state, with the stress stimulus inducing an overaccumulation of ABA-responsive
transcripts in the mutant. Alternatively, the stronger phenotype of scl30a-1 under stress when
compared to control conditions could indicate stress regulation of SCL30a activity. The fact that the SCL30a expression and splicing pattern is unaffected by ABA or salt (data not shown and [34,49]) points to posttranslational regulation of this RNA-binding protein. In support of this notion, SR proteins are known to undergo extensive phosphorylation at their RS domain [14], and stress cues affect both the phosphorylation status and activity of Arabidopsis SR and SR-related proteins [23,61–65]. Notably, SR protein kinase 4 (SRPK4) and stress-responsive mitogen-activated protein kinases (MAPKs) were found to phosphorylate SCL30, a close SCL30a paralog [66].

Quite surprisingly, our large-scale transcriptome analysis revealed only 22 alternative splicing events in 21 genes affected in the scl30a-1 mutant (dPSI > |15|), thus precluding solid mechanistic insight into the splicing function of this SR protein. Our results contrast with a main expected role for SCL30a as a splicing regulator and raise the question of whether this protein is involved in regulating other steps of gene expression. Beyond splicing, animal SR proteins have been shown to play important roles in coordinating several steps of gene expression, including transcriptional activation, nonsense-mediated decay, mRNA export and translation [18,19,67]. In Arabidopsis, SCL proteins can interact with the NRPB4 subunit of the RNA Polymerase II, pointing to a potential role in the regulation of gene transcription, and simultaneous disruption of the four SCL subfamily genes and SC35 causes drastic transcriptional changes [33]. Therefore, and in alignment with our transcriptomic results, an important component of SCL30a function during seed germination could lie in the regulation of gene transcription. Nonetheless, the RNA-seq experiment performed here reflects the transcriptome of scl30a-1 germinating seeds at a specific time point (18 hours after stratification), and the possibility that the observed gene expression changes are a consequence of an earlier alternative splicing defect cannot be ruled out. Future identification of the direct
targets of SCL30a using immunoprecipitation methods should provide insight into the molecular functions of this protein during seed germination and stress responses.

Seed size is a major component of crop yield and salt stress dramatically reduces plant productivity worldwide. We have disclosed a novel function for an Arabidopsis splicing factor—SCL30a—in governing seed size and tolerance to salt stress. Our data also suggest a non-canonical role for the SCL30a protein, where it could regulate gene transcription rather than alternative splicing during seed germination. Moreover, we provide evidence that SCL30a modulates seed traits by interacting with the ABA pathway. The larger and salt-tolerant seeds produced by SCL30a-overexpressing plants underscore the high potential of this protein for biotechnical applications. Deeper insight into the mode of action of SCL30a may translate into improved crop performance under adverse environmental conditions.

Materials and methods

Plant materials and growth conditions
The Arabidopsis thaliana ecotype Colombia (Col-0) was used as the wild type in all experiments. Seeds were surface-sterilized for 10 minutes in 50 % (v/v) bleach and 0.07% (v/v) TWEEN®20, stratified for 3 days at 4 ºC in the dark and plated on MS media [1X Murashige and Skoog (MS) salts (Duchefa Biochemie), 2.5 mM MES (pH 5.7), 0.5 mM myo-inositol and 0.8 % (w/v) agar], before transfer to a growth chamber under 16-h photoperiod (long-day conditions) or continuous light (cool white fluorescent bulbs, 18W840, 4000K at 100 µmol m⁻² s⁻¹) at 22 ºC (light period) or 18 ºC (dark period) and 60 % relative humidity. Seed imbibition (Fig 1, 2A, 5B and 6C) was always performed at 4 ºC (equivalent to stratification). After 2-3 weeks, seedlings were transferred to soil in individual pots.

PCR-based genotyping of the SALK_041849 line (obtained from NASC) with primers specific for SCL30a and the left border of the T-DNA (S7 Table) followed by sequencing of
the genomic DNA/T-DNA junction confirmed the insertion site and allowed isolation of a homozygous line, which was backcrossed twice with the wild type. The scl30a-1 mutant was independently crossed with the aba2-1 [6] and the abi4-101 [57] alleles (obtained from NASC) and the corresponding double mutants identified via PCR screening (S7 Table) of F2 progeny following F1 self-fertilization.

**Generation of transgenic plants**

Plant transformation was achieved by the floral dip method [68] using *Agrobacterium tumefaciens* strain EHA105. For reporter gene experiments, the 2206 bp immediately upstream of the SCL30a start codon were PCR-amplified (S7 Table) from genomic DNA and subcloned into the pGEM vector (Promega), where the eGFP-GUS segment isolated from the pKGWFS7 vector [69] using the SacII/NcoI restriction sites was fused at the 3’ end of the SCL30a promoter sequence. The entire fragment was transferred into pKGWFS7 via the SpeI/NcoI restriction sites, replacing the original CmR-ccdB-eGFP-GUS cassette, and the construct agroinfiltrated into Col-0 plants.

To generate the Pro35S:SCL30a.1 construct, an RT-PCR fragment corresponding to the SCL30a.1 transcript (S7 Table) was inserted into the pBA002 backbone using the AscI/PacI restriction sites, and the construct agroinfiltrated in Col-0 plants. Two independent SCL30a-OX lines were first isolated and analyzed. After several seed-to-seed cycles, expression of the transgene in these SCL30a-OX2 and SCL30a-OX3 lines was silenced, with consequent loss of the corresponding phenotypes. A third overexpression line, SCL30a-OX1, was then generated and phenotypically characterized.

**Seed measurements and composition**
Wild-type (Col-0) and mutant (scl30a-1) plants were sown and grown to maturity simultaneously under identical conditions, and all assays were performed with seeds from comparable lots.

The area of dry and imbibed seeds was measured using the ImageJ software (http://rsbweb.nih.gov/ij). To determine seed weight, six groups of 1000 dry seeds were weighed using an Acculab ALC-80.4 (Sartorius) analytical balance.

For compositional analysis, dry seeds were bulk harvested by genotype and homogenized. The oil, protein and soluble carbohydrate contents were determined as described previously [70]. To analyze fatty acids, dry seeds (20 mg) were crushed and sonicated in 2 mL of heptane for 15 minutes at 60 °C. After centrifuging for 5 minutes at 2,000 g, 200 µL of the heptane layer were transferred to a small vial with 50 µL of trimethylsulfonium hydroxide (TMSH) in methanol and an additional 300 µL of heptane. After incubation for 30 minutes at room temperature, 1 µL of the upper heptane layer was used to analyze the fatty acid methyl esters, which were separated and quantified using a Hewlett-Packard 6890 gas chromatograph as described in Cahoon et al. (2001). All analyses were performed in duplicate on three independent seed batches per genotype.

**Germination and dormancy assays**

For germination assays, fully mature siliques from dehydrated plants were collected and stored in the dark at room temperature for at least one week before phenotypical analysis. After surface-sterilization and stratification for 3 days at 4 °C in the dark, 70-100 seeds of each genotype were sown on MS media supplemented or not with the appropriate concentrations of NaCl, ABA (mixed isomers, A1049; Sigma) or fluridone (45511, Fluka) and then transferred to long-day conditions, except for the determination of germination rates under control conditions (Fig 2C and 5C), which was conducted under continuous light to avoid the effect of long dark periods during a short time course. To assess dormancy, seeds from freshly mature
siliques were collected from the tree, immediately surface-sterilized and plated on MS media before transfer to dark at 22 ºC, with control seeds being stratified for 3 days at 4 ºC in the dark before transfer to long-day conditions. Percentages of seed germination, defined as protrusion of the radicle through the seed coat, were scored over the total number of seeds. The results presented are representative of at least three independent experiments.

ABA content determination

Mature seeds harvested from Col-0, scl30a-1, SCL30a-OX2 or aba2-1 dehydrated plants and stored for 5 months were stratified for 3 days at 4 ºC in the dark, sown on MS media with or without 200 mM NaCl and grown for 2 days under long-day conditions. Seeds were then collected and endogenous ABA levels quantified using an immunoassay as described in [26].

Expression and alternative splicing analyses of individual genes

Histochemical staining of GUS activity in ProSCL30a:GUS transgenic lines was performed as described by Sundaresan et al. [71].

For the RT-PCR analysis shown in Fig 1, S1 Fig and S3 Fig, total RNA was extracted from different plant tissues using TRI Reagent (T924; Sigma-Aldrich) or from dry, imbibed and up to 5-day germinated seeds using the innuPREP Plant RNA Kit (Analytik Jena). First-strand cDNA synthesis and PCR amplification were performed as described in [72], using the primers and number of cycles indicated in Table S7 as well as ROC1 as a reference gene. Results are representative of at least three experiments.

For the RT-qPCR analyses shown in Fig 2D, seeds were stratified for 3 days at 4 ºC, sown on MS media, transferred to continuous light conditions, and collected after 18 hours (prior to radicle emergence) to avoid major developmental effects. For the RT-qPCR of Fig 5A, seedlings were grown for 1 week after stratification. Total RNA was extracted using the innuPREP Plant RNA Kit (Analytik Jena), digested with the RQ1 DNase (Promega), and first
strand cDNA synthesized using 1 µg RNA, Super Script III Reverse Transcriptase (Invitrogen) and a poly-T primer. qPCR was performed using an ABI QuantStudio sequence detection system (Applied Biosystems) and Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific) on 2.5 µL of cDNA (diluted 1:10) per 10-µL reaction volume, containing 300 nM of each gene-specific primer (S7 Table). Reaction cycles were 95 ºC for 2 min (1X), 95º C for 30 s/60 ºC for 30 s/72 ºC for 30 s (40X), followed by a melting curve step to confirm the specificity of the amplified products. UBQ10 and ROC5 were used as reference genes. Each experiment was replicated at least three times.

For the analyses of alternative splicing shown in S2 Fig, PCR with the NZYTaq II 2x Green Master Mix (Nzytech) was performed on cDNA from three biological replicates of germinating seeds (18 hours after stratification, continuous light) using primers flanking the alternatively spliced intron (S7 Table) obtained from PASTDB (pastdb.crg.edu). Reaction cycles were 95 ºC for 3 min (1X), 95 ºC for 30 s/58 ºC for 30 s/72 ºC for 5 min (35X). The PCRs products then were loaded on a 2% agarose gel and gel bands quantified using the ImageJ software (http://rsbweb.nih.gov/ij). The percent spliced-in (PSI) for each alternative splicing event was calculated after quantification of the inclusion (I) or splicing (S) for a given event as PSI = I / (I + S).

**RNA-seq sample preparation and sequencing**

Approximately 50 mg of Col-0 wild type and scl30a-1 mutant seeds (three biological replicates) were surface-sterilized, stratified at 4 ºC for 3 days and sown on MS media for 18 hours under continuous light, before total RNA was extracted using the innuPREP Plant RNA Kit (Analytik Jena). The RNA-seq libraries generated from Col-0 and scl30a-1 seeds were prepared and sequenced at the Center for Genomic Regulation (Barcelona, Spain) using the HiSeq Sequencing V4 Chemistry kit (Illumina, Inc) and the HiSeq 2500 sequencer (Illumina, Inc), with a read length of 2 x 125 bp.
RNA-seq quantification of sequence inclusion and identification of differentially-spliced genes

We employed vast-tools v2.5.1 to quantify alternative splicing from RNA-seq for *A. thaliana* [36,73]. This tool quantifies exon skipping (ES), intron retention (IR), and alternative 3’ (Alt3) and 5’ (Alt5) splice sites. For all these types of events, vast-tools estimates the percent of inclusion of the alternative sequence (PSI) using only exon-exon (or exon-intron for IR) junction reads and provides information about the read coverage (see https://github.com/vastgroup/vast-tools for details). To identify alternative splicing events regulated by SCL30a we used vast-tools compare. This function compared PSI values of each AS event with sufficient read coverage in all wild-type and *scl30a-1* samples being tested (three biological replicates of each genotype) and selected those with an average ΔPSI > 15 and a ΔPSI between the two distributions > 5 (--min_dPSI 15 --min_range 5) (see https://github.com/vastgroup/vast-tools and [73] for details). We also used the --p_IR filter to discard introns with a significant read imbalance between the two exon-intron junctions (p < 0.05, binomial test; see [74] for details). Moreover, to ensure that Alt3 and Alt5 are located in exons with a sufficient inclusion level, we used the option –min_ALT_use 25, which implies that the host exon has a minimum PSI of at least 25 in each analyzed sample.

RNA-seq quantification of gene expression and identification of differentially expressed genes

Quantification of Arabidopsis transcript expression from our RNA-seq experiment and public sequencing data on seed germination (GSE94459) was performed using vast-tools v2.5.1 [73]. This tool provides cRPKMs numbers for each Arabidopsis transcript as the number of mapped reads per million mapped reads divided by the number of uniquely mappable positions of the transcript [36]. To identify differentially expressed genes between wild-type and *scl30a-1* germinating seeds, we used vast-tools compare_expr using the option -norm (see
https://github.com/vastgroup/vast-tools for details). In brief, a quantile normalization of cRPKM values with “Normalize Between Arrays” within the “limma” package of R is first performed. Next, genes that were not expressed at cRPKM > 5 are filtered out and read counts > 50 across all the replicates of at least one of the genotypes compared. Graphs in Fig 3 and 4 only show expression of genes that passed these cut-offs. Finally, differentially-expressed genes were defined as those with a fold change of at least 2 between each of the individual replicates from each genotype.

Assessment of overlap between SCL30a- and ABA-regulated genes

ABA-regulated genes were obtained from the reanalysis of GSE62876 [49] using the default settings of GEO2R (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html). Three comparisons were conducted: 0 hours of ABA treatment versus 2, 12 or 24 hours. Genes regulated at any of these timepoints were selected (FC > 2; adjusted p-value < 0.05). Given that ABA-regulated genes in [49] are defined based on microarray studies, which do not assess expression of all Arabidopsis genes as RNA-seq experiments do, for the comparison we discarded one SCL30a-regulated gene not represented in the microarray. We also discarded ABA-regulated genes not expressed in our RNA-seq samples (see previous section).

Gene ontology enrichment analyses

The Gene Ontology (GO) enrichment analysis shown in Fig 4, which identifies significantly enriched biological processes, molecular functions and cellular components among the genes up- and downregulated in the scl30-1 mutant, was performed using the functional annotation classification system DAVID version 6.8 [75]. Only statistically significant GO categories (p < 0.05) are shown in Table S3.

Accession numbers
Raw sequencing data and transcript expression results were submitted to the Sequence Read Archive (accession number GSE181122).

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**Figure Legends**

**Fig 1. SCL30a promoter activity and expression pattern in Arabidopsis.**

(A) Differential interference contrast microscopy images of GUS-stained transgenic plants carrying the promSCL30a:GUS reporter construct. SCL30a promoter activity in 2-week-old seedlings (a), the primary root tip (b), a lateral root primordium (c), mature and immature flowers (d), developing embryos (e-j), the embryo (k) and testa (l) from imbibed mature seeds, and seeds germinated for 1-2 days (m). Scale bars, 100 µm. (B) RT-PCR analysis of SCL30a transcript levels in vegetative and embryonic tissues of wild-type (Col-0) plants. The location of the F1 and R1 primers is shown in S1A Fig. Expression of the cyclophilin (ROC1) gene was used as a loading control.

**Fig 2. Effect of the scl30a-1 mutation on seed size, dormancy, germination and seed development gene expression.**
(A) Representative images of dry and imbibed wild-type (Col-0) and mutant (scl30a-1) seeds (scale bars, 1.5 mm), and quantification of the area of Col-0 (white bars) and scl30a-1 (black bars) seeds (means ± SE, n ≥ 60). (B) Germination percentages of freshly-harvested Col-0 (white bars) and scl30a-1 (black bars) seeds scored upon either stratification and 7 days of incubation in light (control) or no stratification and 7 days of incubation in darkness (means ± SE, n = 3). (C) Germination rates of Col-0 (white circles) and scl30a-1 (black circles) seeds scored during the first 3 days after stratification (means ± SE, n = 3). (D) RT-qPCR analysis of the expression levels of the ABI3, ABI5, Em1, Em6 and LEA4-5 genes in Col-0 (white bars) and scl30a-1 (black bars) seeds 18 hours after stratification (means ± SE, n = 4). Expression of the cyclophilin (ROC5) gene was used as a loading control. In A-D, asterisks indicate significant differences from the Col-0 wild type (* p < 0.05, ** p < 0.01, *** p < 0.001; Student’s t-test).

Fig 3. Genes differentially expressed in the scl30a-1 mutant and their expression pattern during seed germination.

Box plot representations of the expression levels of the 315 genes upregulated (A) or of the 67 genes downregulated (B) in the scl30a-1 mutant (left) and their expression values in samples collected at different stages of seed germination obtained from [9] (right). See Materials and methods for details.

Fig 4. Gene ontology analysis of scl30a-1-regulated genes and overlap with ABA transcriptional responses.

(A-B) The ten most significantly enriched gene ontology categories, including biological process (purple bars), cellular component (dark-green bars) and molecular function (light-green bars), for the genes up- (A) and down- (B) regulated in the scl30a-1 mutant. (C-D) Overlap between the genes up- (C) or down- (D) regulated in the scl30a-1 mutant (green
circles) with those up- (C) or down- (D) regulated by ABA in Arabidopsis germinating seeds from Costa et al. [49] (blue circles), respectively (see Materials and methods for details). The boxplots on the right represent the distribution of expression of the 1446 ABA-upregulated genes (C) and 1675 ABA-downregulated genes (D) [49] in wild-type (Col-0) and scl30a-1 mutant germinating seeds (see Materials and methods for details), with the asterisks indicating significant differences from the Col-0 wild type (Wilcoxon test, *** p < 0.001).

Fig 5. Seed and germination phenotypes conferred by SCL30a overexpression.

(A) RT-qPCR analysis of the expression levels of SCL30a in Col-0 (white bar), scl30a-1 (black bar) and SCL30a-OX1 (grey bar) 7-day-old seedlings (means ± SE, n = 4). Expression of the ubiquitin (UBQ10) gene was used as a loading control. (B) Size (expressed as area) of imbibed Col-0 (white bar), scl30a-1 (black bar) and SCL30a-OX1 (gray bar) seeds (means ± SE, n ≥ 30). (C) Germination rates of Col-0 (white circles), scl30a-1 (black circles), and SCL30a-OX1 (gray circles) seeds scored during the first 3.5 days after stratification (means ± SE, n = 3). (D) Representative images of Col-0, scl30a-1 and SCL30a-OX1 seeds germinating in the absence (control) or presence of 5 μM ABA or 200 mM NaCl 7 days after stratification, and germination percentages of Col-0 (white bars), scl30a-1 (black bars) and SCL30a-OX1 (gray bars) seeds in the absence (control) or presence of 5 μM ABA or 200 mM NaCl scored 7 days after stratification (means ± SE; n = 3). In A-D, asterisks indicate statistically significant differences from the Col-0 wild type (* p < 0.05, ** p < 0.01, *** p < 0.001; Student’s t-test).

Fig 6. ABA dependence of the scl30a-1 mutant phenotypes.

(A) Germination percentages of Col-0 (white bars) and scl30a-1 (black bars) seeds in the absence (control) or presence of 200 mM NaCl supplemented or not with 1 µM fluridone, scored 5 days after stratification (means ± SE, n = 3). Asterisks indicate statistically significant differences from the Col-0 wild type (** p < 0.01; Student’s t-test). (B) Germination rates of
Col-0, scl30a-1, aba2-1, abi4-101, scl30a-1 aba2-1 and scl30a-1 abi4-101 seeds under different NaCl concentrations scored 4 days after stratification (means ± SE, n = 3). Asterisks indicate statistically significant differences between the scl30a-1 mutant and the Col-0 wild type or the double mutants and the corresponding ABA single mutant (* p < 0.05, ** p < 0.01, *** p < 0.001; Student’s t-test). (C) Size (expressed as area) of imbibed Col-0, scl30a-1, aba2-1, abi4-101, scl30a-1 aba2-1 and scl30a-1 aba4-101 seeds (means ± SE, n ≥ 60). Asterisks indicate statistically significant differences from the Col-0 wild type (*** p < 0.001; Student’s t-test). (D) Germination percentages of freshly-harvested Col-0, scl30a-1, aba2-1, abi4-101, scl30a-1 aba2-1 and scl30a-1 abi4-101 seeds scored upon either stratification and 7 days of incubation in light or 7 days of incubation in darkness (means ± SE, n = 3). Asterisks indicate statistically significant differences from the Col-0 wild type (*** p < 0.001; Student’s t-test).

Supporting Information Legends

S1 Fig. Structure of the SCL30a gene and isolation of the scl30a-1 loss-of-function mutant.

(A) Schematic representation of the SCL30a gene showing the site of insertion and orientation of the T-DNA in the scl30a-1 mutant (boxes indicate exons with UTRs in grey, lines between boxes represent introns, and arrows indicate the location of SCL30a- and T-DNA-specific primers), and structure of the three identified splice variants as well as of the corresponding predicted protein isoforms (RRM, RNA recognition motif; RS, arginine/serine-rich domain). The asterisks mark the position of the predicted protein truncation in the scl30a-1 mutant. (B) RT-PCR analysis of SCL30a transcript levels in wild-type (Col-0) and mutant (scl30a-1) 5-day old seedlings using primers flanking the T-DNA, and up- or downstream of the insertion site. The location of the F1, F3, R1 and R2 primers is shown in (A). The UBIQUITIN 10 (UBQ10) gene was used as a loading control.
S2 Fig. Validation of selected differential alternative splicing events detected by RNA-seq.

RT-PCR analysis of individual AS events differentially regulated between Col-0 and scl30a-1 germinating seeds 18 hours after stratification in (A) AT5G64980 (event: AthINT0051338), (B) AT3G07890 (event: AthINT0022974), (C) AT2G46915 (event: AthINT0021222) and (D) AT5G09690 (event: AthINT0086682). Graphs represent percent spliced-in (PSI) values (means ± SE n = 3-5) after quantification of the corresponding band intensities using the Image J software. Asterisks indicate statistically significant differences from the Col-0 wild type (* p < 0.05; Student’s t-test).

S3 Fig. Characterization of the SCL30a-OX2 and SCL30a-OX3 overexpression lines.

(A) RT-PCR analysis of SCL30a transcript levels in 7-day-old seedlings of Col-0, scl30a-1 and two SCL30a overexpression lines (SCL30a-OX2 and SCL30a-OX3). The location of the F2 and R1 primers is shown in S1A Fig. The UBIQUITIN 10 (UBQ10) gene was used as a loading control. (B) Size (expressed as area) of imbibed Col-0 (white bars), scl30a-1 (black bars) and SCL30a-OX2 or SCL30a-OX3 (gray bars) seeds (means ± SE, n ≥ 60). (C) Germination percentages of Col-0 (white bars), scl30a-1 (black bars) and SCL30a-OX2 or SCL30a-OX3 (gray bars) in the absence (control) or presence of 3 µM ABA or 200 mM NaCl scored 5 days after stratification. Bars represent means ± SE, n = 3. In B and C, asterisks indicate significant differences from the Col-0 wild type (* p < 0.05, ** p < 0.01, *** p < 0.001; Student’s t-test).
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