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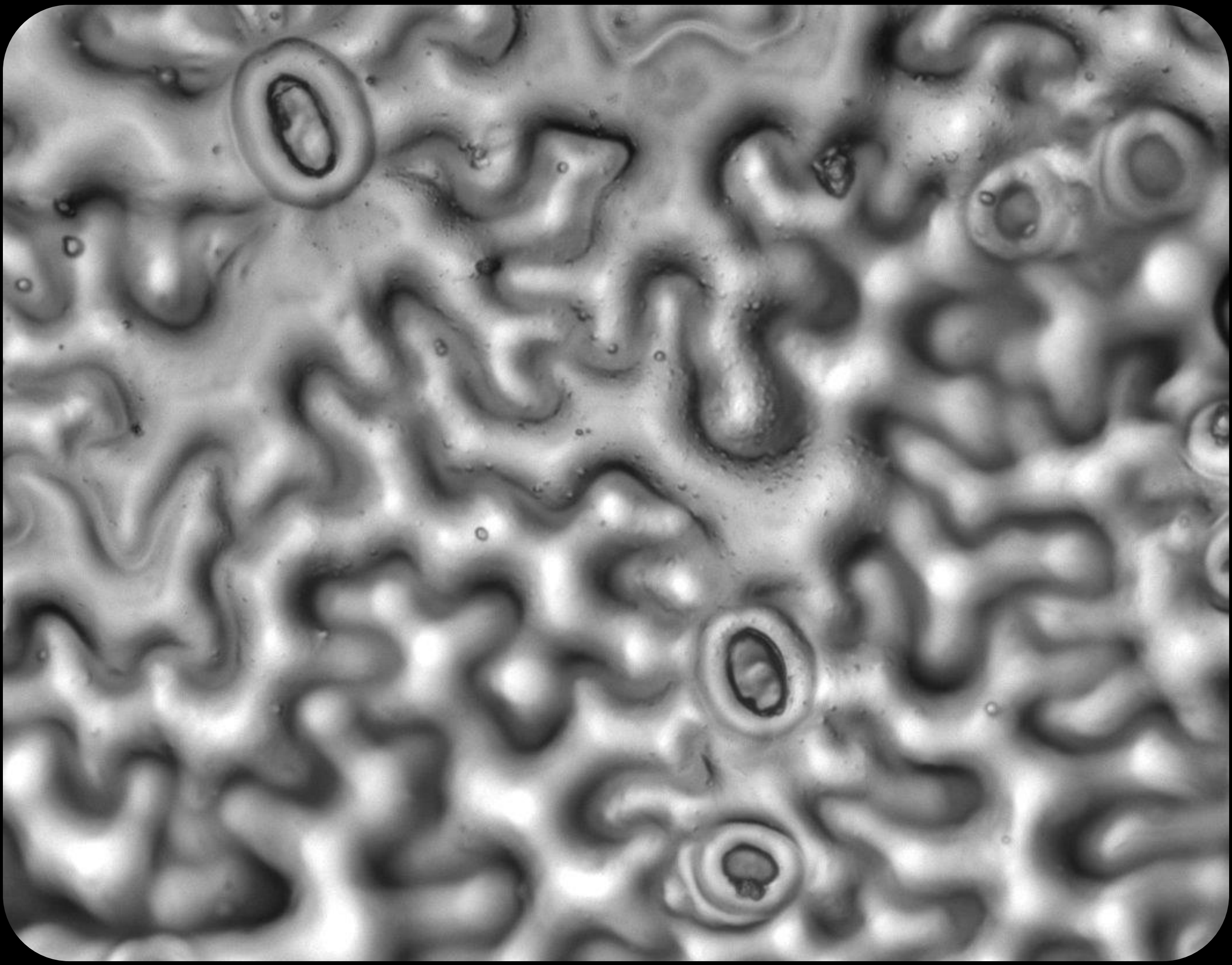
Contribution of Alternative Splicing to the ABA Response

Insights from Natural Variants

Alba Rodríguez Díez



Dissertation presented to obtain the **Ph.D degree in Integrative Biology and Biomedicine**

Oeiras, October, 2024

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER | UNIVERSIDADE NOVA DE LISBOA

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DECLARATION

This dissertation and data contained are result of my own work conducted between 2017 and 2024 in the lab of Dr. Paula Duque at the Instituto Gulbenkian de Ciência (Oeiras, Portugal). Financial support for this work was granted to Alba Rodríguez Díez by Fundação para a Ciência e Tecnologia (FCT) through doctoral fellowship SFRH/PD/BD/114348/2016.

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RESUMO

O “splicing” alternativo surgiu recentemente como um fator significativo na tolerância das plantas ao stress, mas os genes e eventos específicos envolvidos permanecem em grande parte desconhecidos. A hormona vegetal ácido abscísico (ABA) é um regulador crucial das respostas das plantas ao stress, orquestrando as reações ao stress abiótico e biótico por meio de uma complexa via de sinalização. A sinalização do ABA pode desencadear respostas rápidas, como o fecho dos estomas, e induzir alterações a longo prazo na expressão dos genes e na regulação pós-transcricional. Em particular, o “splicing” alternativo começa a ser reconhecido como um mecanismo importante nas respostas das plantas ao stress, tendo como alvo a via do ABA. No meu projeto de doutoramento, utilizei a variância natural na planta modelo *Arabidopsis thaliana* para fundamentar o papel do “splicing” alternativo nas respostas ao stress mediadas pela fito-hormona ABA e para descobrir alvos de “splicing” funcionalmente significativos.

O primeiro objetivo do meu estudo foi identificar variantes naturais de *A. thaliana* com diferentes sensibilidades ao ABA (Capítulo 2). Concebi um rastreio fenotípico preliminar utilizando 24 ecótipos de *A. thaliana*. Estes ecótipos foram submetidos a diferentes tratamentos com várias concentrações de ABA, em diversos tecidos e fases de desenvolvimento da planta, incluindo ao nível dos estomas. Embora as respostas estomáticas tenham sido amplamente estudadas, os métodos simples para a sua avaliação eram limitados. Esta tese apresenta um protocolo otimizado para avaliar as respostas ao ABA ao nível dos estomas, utilizando um método simples, económico e duradouro (Capítulo 3).

A partir do rastreio inicial, a Kn-0 surgiu como uma variante natural com uma sensibilidade reduzida ao ABA em comparação com o ecótipo de referência Col-0, totalmente anotado. Esta diferença na resposta fisiológica foi ainda corroborada a nível molecular pela indução reduzida de genes marcadores de ABA no ecótipo Kn-0 após tratamento transitório com a fito-hormona. A análise por RNA-seq de plantas Col-0 e Kn-0 cultivadas em condições de controlo e de ABA foi então realizada para identificar alterações na expressão génica e no “splicing” alternativo associadas à sensibilidade ao ABA (Capítulo 4). Em geral, observámos menos alterações na expressão génica e no “splicing” alternativo no ecótipo menos sensível, Kn-0, com uma divergência mais pronunciada no “splicing” alternativo. Nomeadamente, foram encontradas diferenças na expressão de genes-chave envolvidos na biossíntese e sinalização de ABA, bem como variações no conteúdo endógeno de ABA. Estas alterações podem ser atribuídas a polimorfismos no promotor ou na região codificante destes genes. Além disso, identificámos alvos promissores de “splicing” alternativo envolvidos na acumulação de ABA através de biossíntese, o que poderá explicar a resposta diferencial à hormona.

De um modo geral, o “splicing” alternativo parece desempenhar um papel complementar mas mais específico do ecótipo relativamente à expressão génica na formação das respostas ao ABA, especialmente no contexto da variação natural. No Capítulo 5, é discutido como os nossos resultados sublinham um papel importante do “splicing” alternativo na regulação das respostas ao ABA e a sua contribuição para a variação natural destas respostas. Em conclusão, esta tese destaca o papel crucial do “splicing” alternativo na modulação das respostas ao ABA e o seu impacto na variação natural observada entre diferentes ecótipos de Arabidopsis, oferecendo novas perspetivas sobre a natureza complexa das respostas das plantas ao stress.

ABSTRACT

Alternative splicing has emerged as a significant contributor to plant stress tolerance, yet the specific genes and events involved remain largely elusive. The plant hormone abscisic acid (ABA) serves as a key regulator of plant stress responses, orchestrating reactions to both abiotic and biotic stress through its intricate signalling pathway. ABA signalling can trigger rapid responses, such as stomatal closure, and induce longer-term changes in gene expression and post-transcriptional regulation. In particular, alternative splicing is being recognized as an important mechanism in plant stress responses by targeting the ABA pathway. In my PhD project, I used natural variance in the model plant *Arabidopsis thaliana* to substantiate a role for alternative splicing in stress responses mediated by the ABA phytohormone and to uncover functionally significant splicing targets.

The first objective of my study was to identify *A. thaliana* natural accessions with varying sensitivities to ABA (Chapter 2). I designed a preliminary phenotypic screen using 24 *A. thaliana* accessions, subjecting them to treatments with different ABA concentrations, across various tissues and developmental stages, including at the stomatal level. Although stomatal responses have been extensively studied, simple methods for their assessment were limited. This thesis introduces an optimised protocol for evaluating ABA responses at the stomatal level, employing a simple, cost-effective, and durable method (Chapter 3).

From the initial screening, Kn-0 emerged as a natural variant with reduced sensitivity to ABA compared to the fully annotated reference ecotype Col-0. This difference in physiological response was further supported at the molecular level by the reduced induction of ABA marker genes in the Kn-0 ecotype following transient treatment with the phytohormone. RNA-seq analysis of Col-0 and Kn-0 accessions grown under both control and ABA conditions was then performed to identify changes in gene expression and alternative splicing associated with ABA sensitivity (Chapter 4). Overall, we observed fewer changes in both gene expression and alternative splicing in the less sensitive Kn-0 ecotype, with a more pronounced divergence in alternative splicing. Notably, differences were found in the expression of key genes involved in ABA biosynthesis and signalling, as well as variations in the endogenous ABA content. These changes may be attributed to polymorphisms in the promoter or coding region of these genes. Furthermore, we identified promising alternative splicing targets involved in ABA accumulation via biosynthesis, which could explain the differential response to the hormone.

Overall, alternative splicing appears to play a complementary but more ecotype-specific role compared to gene expression in shaping ABA responses, especially in the context of natural variation. Chapter 5 discusses how our results underscore a major role for alternative splicing in regulating ABA responses and its contribution to the natural variation of these responses. In conclusion, this thesis highlights the crucial role of alternative splicing in modulating ABA responses and its impact on the natural variation observed between different Arabidopsis ecotypes, offering new insights into the complex nature of plant stress responses.

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And here we are. I'd be lying if I said I never doubted I'd get to write these pages. This book represents eight years of my life, from 2016 to 2024. This journey has taken me through many phases, as it does for many students. I began with passion, excitement, and love for this career, and I leave exhausted, relieved that it comes to an end, and with the fear of not having met everyone's expectations. But I would do it all again and have many people to thank for it.

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My love for science was not something I was born with but rather something cultivated by my parents and grandparents. They surrounded my brother and me with books, took us to museums, encouraged our curiosity, and never imposed us any barriers – we were free to explore. They fought and made tremendous efforts to give us the best education possible, and I'd like to think that their hard work has paid off. My deepest thanks go to them. They have been my unwavering support, always there for me, no questions asked, even in the toughest moments. I also want to remember my mom – you are still with us, but it is not you anymore. I know you would have been very proud of me, and this journey would have been so much easier if you were here. You were my role model: intelligent, kind, loving, and caring. This goes to you and dad, my other cornerstone. Papá, this house would not have sustained itself without you. Thank you for always believing in me. To my brother Iago, for having to 'deal' with me his whole life, always cheerful and optimistic – thank you for your constant support over these years. To my grandmothers, Tata Fina and Tata Vita, the strongest women I know. To my big sister and confidante, Iria, I don't know what I would do without you. To my cousins, aunts, uncles, *padrinos*, and those no longer with us – thank you for your constant help and support. You kept me going. To my chosen family – Dani, Lucía – you have been my safe space since the beginning, and can't thank you enough for your support, here is to another 30 years of friendship. Even from a distance, you were and are always there. This also goes to Borjis, Pablo, and Nick, your support has helped me reach the end.

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This thesis has been a wild ride. Thanks for reading it.

Alba

LIST OF ABBREVIATIONS

A3SS	Alternative 3' splice site
A5SS	Alternative 5' splice site
AAO3	Abscisic aldehyde oxidase 3
ABA	Abscisic acid
ABA-GE	ABA-glucosyl ester
ABA2/3/4	ABA-deficient 2/3/4
ABCC1/2	ATP-binding cassette class C 1/2
ABCG17/18/25/30/40	ATP-binding cassette class G 17/18/25/30/40
ABH2	ABA hypersensitive 2
ABI1-5	ABA insensitive 1-5
ABT	ABA signalling terminator
AEL	Arabidopsis EL1-like
AHG1	ABA hypersensitive germination 1
AHG3/AtPP2CA	ABA hypersensitive germination 3/Protein phosphatase 2CA
ALDH7B4	Aldehyde dehydrogenase 7B4
ALIX	ALG-2 interacting protein-X
AP2/ERF	APETALA / Ethylene responsive binding factor
AREB/ABF	ABA-responsive element (ABRE) binding protein/ABRE-binding factor
AREB2/ABF4	ABA-responsive element (ABRE) binding protein 2/ABRE-binding factor 4
AtACINUS	Arabidopsis homolog of mammalian apoptotic chromatin condensation inducer in the nucleus
AtHKT1	*Arabidopsis thaliana* high-affinity K^+ transporter 1
BAK1	BRI1-associated receptor kinase 1
BAM	Barely any meristem
BG1/2	β-glucosidase 1/2
BGLU10	β-glucosidase 10
bHLH122	Basic helix-loop-helix 122
BIN2	Brassinosteroid insensitive 2
BRM	BRAHMA
BTR1L	Binding to ToMV RNA 1, large protein product
bZIP	Basic leucine zipper-type protein

CARK1	Cytosolic ABA receptor kinase 1
CDS	Coding sequence
CEPR2	C-terminally encoded peptide receptor 2
CK	Cytokinin
CK2	Casein kinase 2
CLE25	CLAVATA3/Embryo surrounding region-related 25
COR	cold-responsive
CPK	Calcium-dependent protein kinase
CYP707A	Cytochrome P450 family 707 subfamily A
DDA1	DET1-DDB1-associated 1
DPA	Dihydrophaseic acid
DPAG	DPA-4-O-β-D-glucoside
DREB	Drought response element binding
DRT111	DNA-damage repair/toleration protein 111
DTX/MATE	Detoxification efflux carriers/Multidrug and toxic compound extrusion
EAR1	Enhancer of ABA co-receptor 1
EGR1/2	Clade E growth-regulator 1/2
eQTL	Expression QTL
ER	Endoplasmic reticulum
ERD	Early responsive to dehydration
ERD15	Early responsive to dehydration 15
ES	Exon skipping
ET	Ethylene
FUS3	FUSCA3
GA	Gibberellic acid
GORK	Guard cell outward-rectifying K^+
GPDHC1	6-phosphogluconate dehydrogenase family protein
GR1	Glutathione reductase 1
GT	Glycosyltransferase
GWAS	Genome-wide association studies
HAB1/2	Homology to ABI1 1/2
HAI1-3	Highly ABA-induced 1-3
HAT1/3	Homeodomain-leucine zipper protein 1/3
hnRNP	heterogeneous nuclear ribonucleoprotein
HOS15	High expression of osmotically responsive genes 15

indel	Insertion and deletion
IR	Intron retention
JA	Jasmonic acid
KAT1	K^+ channel in Arabidopsis thaliana 1
KEG	Keep on going
KIN	Cold-inducible
KUP6	K^+ uptake transporter 6
L1L	LEC1-like
LEA	Late embryogenesis abundant
LEC1/2	Leafy cotyledon 1/2
LHY	Late elongated hypocotyl
LR	Lateral root
LRK10L1	Leaf rust 10 disease-resistance locus receptor-like protein kinase-like1
LRP	Lateral root primordia
LSM1-7	SM-like protein 1-7
LTI	Low temperature-induced
MAP	Mitogen-activated protein
MEP	Methylerythritol phosphate
MoCo	Molybdenum cofactor
mRNA	messenger RNA
MYB	Myeloblastosis
MYC	Myelocytomatosis
NAC	NAM (no apical meristem) ATAF (Arabidopsis transcription activation factor) CUC (cup-shaped cotyledon)
NAC003	NAC domain containing protein 3
NCED3	9-cis-epoxycarotenoid dioxygenase 3
NGA1	NGATHA1
NMD	Nonsense-mediated decay
NPF	Nitrate transporter 1/ peptide transporter family
NSY/ABA4	Neoxanthin via neoxanthin synthase/ABA-deficient 4
SnRK2.6/OST1	SNF1-related protein kinase 2.6/open stomata 1
PA	Phaseic acid
PIR1/PIR2	PP2CA interacting RING finger protein 1/2
PP2-B11	Phloem protein 2-B11
PP2AA3	Protein phosphatase type 2A subunit A3

PP2C	Protein phosphatase type 2C
PP2CA	Protein phosphatase type 2C subunit A
PP6	Protein phosphatase 6
PR	Primary root
PR5K2	PR5 receptor-like kinase 2
pre-mRNA	Precursor-mRNA
PTC	Premature termination codon
PUB12/13	Plant U-box 12/13
PUB22/23	Plant U-box 22/23
PYL1-13	PYR-like 1-13
PYR1	Pyrabactin resistance 1
QTL	Quantitative trait locus
RAB18	Responsive to ABA 18
RAS1	Response to ABA and salt 1
RAV1	Related to ABI3/VP1 1
RBM25	RNA-binding protein 25
RbohF	Respiratory burst oxidase homolog protein F
RCAR	Regulatory component of ABA receptor
RD29A	Responsive to desiccation 29A
RDK1	Receptor dead kinase 1
RGLG1/5	RING domain ligase 1/5
RNA-seq	RNA-sequencing
RNAPII	RNA polymerase II
ROP11	Rho-related protein from plants 11
ROS	Reactive oxygen species
RPK1	Receptor-like protein kinase 1
RSL1	Ring finger of seed longevity 1
SA	Salicylic acid
SAD1	Supersensitive to ABA 1
SCL30	SC35-like splicing factor 30
SKIP	Ski-interacting protein
SLAC	Slow anion channel-associated 1
SNP	Single nucleotide polymorphism
SnRK2.1-2.10	SNF1-related protein kinase 2.1-2.10
SnRK2.6/OST1	SNF1-related protein kinase 2.6 / Open stomata 1
snRNP	Small nuclear ribonucleoproteins

SNS1	SnRK2-substrate 1
sQTL	Splicing QTL
SR	Serine/arginine-rich
SUA	Suppressor of ABI3-5
SVP	Short vegetative phase
TOPP1	Type one protein phosphatase 1
TOR	Target of rapamycin
UGT71B6-B8	UDP-glycosyltransferase 71B6-B8
UGT71C5	UDP-glycosyltransferase 71C5
UTR	Untranslated region
VIP1	VirE2-interacting protein 1
VPS23A	Vacuolar protein sorting 23A
WRKY	WRKY DNA-binding protein
ZEP/ABA1	Zeaxanthin epoxidase/ABA-deficient 1

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Chapter 1. General Introduction

1.1 Abscisic acid

As sessile organisms, plants must undergo extensive physiological and molecular changes throughout their lives to cope with varying environmental conditions that negatively impact their growth and yield. Abiotic stresses such as reduced water availability, high salinity, or extreme temperatures, as well as biotic factors, trigger protective responses through the action of phytohormones — small signalling molecules that coordinate growth and development, allowing plants to adapt to changing conditions. Among these phytohormones, abscisic acid (ABA) is considered the major abiotic stress regulator and is rapidly accumulated by plants upon stress perception.

ABA plays a dual role in plants, functioning under both normal and stressful conditions. In the absence of stress, ABA is key for plant growth and developmental processes, ranging from the seed phase, where it maintains seed dormancy and inhibits germination, to the adult stages, where it regulates growth and stomatal movements (reviewed in Cutler et al., 2010; Finkelstein, 2013). Indeed, ABA-deficient mutants with genes involved in signalling and/or biosynthesis of the hormone affected show severely stunted phenotypes, which can be rescued upon exogenous ABA application (reviewed in Cutler et al., 2010; Finkelstein, 2013). However, when plants encounter unfavourable conditions, ABA reinforces seed dormancy, prevents premature germination, closes stomata, and stalls plant growth to conserve energy and activate stress responses (reviewed in Cutler et al., 2010; Finkelstein, 2013).

1.1.1 ABA signalling pathway

The ABA signalling pathway, shown in Figure 1.1, is composed of three core elements: the ABA PYR (pyrabactin resistance)/PYL(PYR-like)/RCAR (regulatory component of ABA receptor) family of receptor proteins (Ma et al., 2009; Park et al., 2009), the clade A PP2C (type 2C protein phosphatases) coreceptors (Leung et al., 1994; Meyer et al., 1994; Rodriguez et al., 1998), and the subclass III SnRK2 (SNF1-related protein kinase 2) protein kinases (Mustilli et al., 2002; Fujii et al., 2007; Fujii and Zhu, 2009). In the presence of stress, intracellular levels of ABA increase and the molecule is perceived by the soluble cytosolic PYR/PYL/RCAR receptors, which upon interaction with PP2Cs, release the SnRK2s from their inhibition (Fujii et al., 2009; Fujita et al., 2009; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009). The binding of ABA to the receptors forms a ligand-receptor complex which is stabilized when bound to the active site of PP2Cs, blocking their catalytic site and inhibiting their phosphatase action on the SnRK2s (reviewed in Ng et al., 2014). The free SnRK2s are then activated via phosphorylation by other protein kinases as well as by autophosphorylation (Nguyen et al., 2019; Takahashi et al., 2020; Lin et al., 2021; Soma et al., 2023). SnRK2s will subsequently phosphorylate downstream targets, including ion channels and transcription factors (Umezawa et al., 2013; Wang et al., 2013), to activate short- and long-term ABA responses such as stomatal closure or ABA-dependent transcriptional changes. When ABA is not present, PP2Cs act as negative regulators and interact with SnRK2s, dephosphorylating Ser/Thr residues on the activation loop, repressing their kinase function, and blocking ABA signalling (Umezawa et al., 2009).

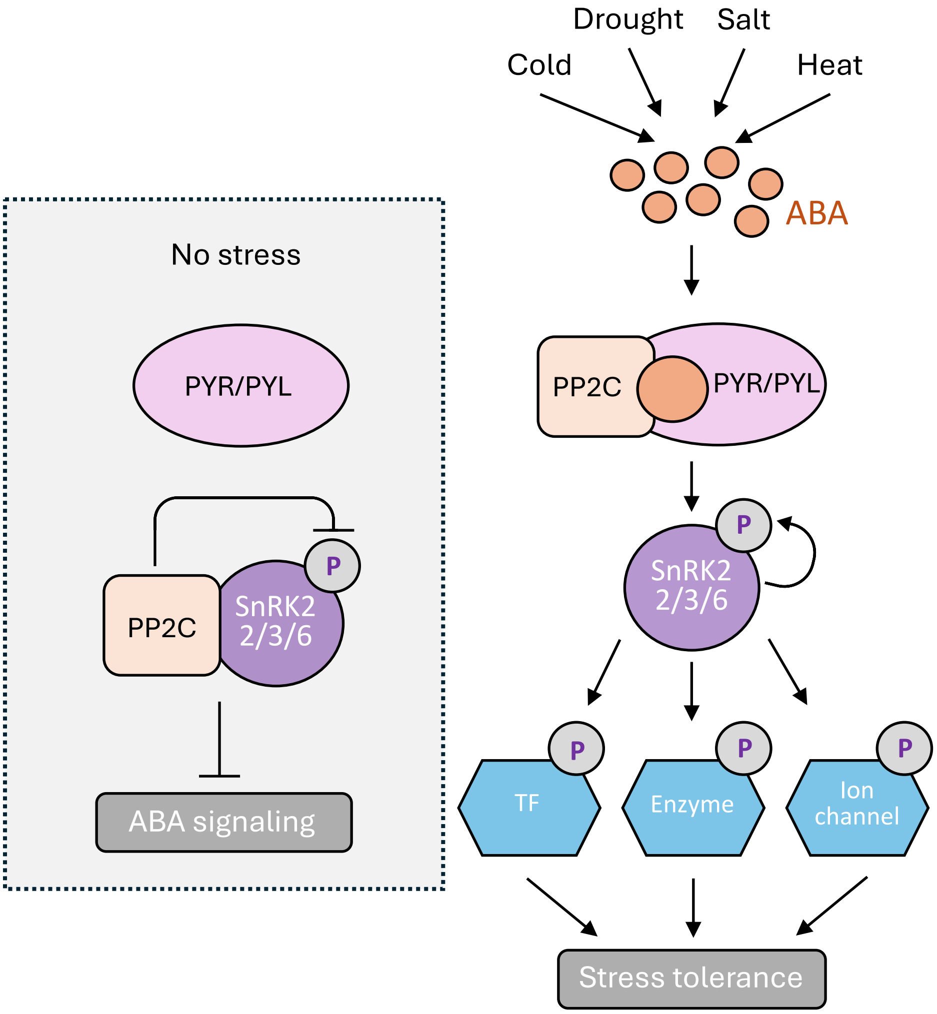

Figure 1.1. The ABA signalling pathway.

Drought, salt, cold and heat trigger the induction of endogenous ABA levels. The hormone binds to the intracellular PYR/PYL receptors, inhibiting PP2C phosphatases. The derepressed SnRK2 kinases then activate downstream targets, helping the plant implement responses to acquire stress tolerance. In the absence of stress, SnRK2s are inhibited by the PP2Cs, and ABA signalling is turned off. Adapted from Laloum et al., 2018.

1.1.1.1 Evolution of the ABA signalling pathway

ABA is ubiquitously found plants but it is also present in organisms across kingdoms from bacteria to animals (reviewed in Olds et al., 2018). The core components of the signalling pathway did not evolve simultaneously. The PP2C-SnRK2 module is highly conserved, while the *bona fide* ABA receptors can only be found in land plants, despite the ABA molecule being already present in algae (reviewed in Umezawa et al., 2010; Cuming, 2019; Komatsu et al., 2020). This suggests, first, that ABA must have had other so far unknown functions in ABA-non-responsive organisms and, second, that ABA must have played an important role in terrestrial colonization of plants, considering it is a crucial molecule for plant dehydration responses and water status control (reviewed in Umezawa et al., 2010; Cuming, 2019; Komatsu et al., 2020).

1.1.1.2 Receptors

In *Arabidopsis thaliana,* the PYR/PYL/RCARs family of receptors (hereafter referred to as PYR/PYLs) is comprised of 14 members: 13 ABA receptors – PYR1 and PYL1-PYL12 – and PYL13, an ABA-irresponsive PP2C regulator (Ma et al., 2009; Park et al., 2009; Li et al., 2013), which can be grouped into three subfamilies based on their sequence similarity. Receptors from subfamilies I and II are monomeric and their members show a high and moderate ABA response, respectively, while in subfamily III they are dimeric and poorly ABA-responsive (Dupeux et al., 2011; Hao et al., 2011; Tischer et al., 2017). Monomeric receptors, such as PYL4-6 and PYL8-10, have higher binding affinity for ABA, require less hormone to inhibit PP2Cs, and some are able to interact and inhibit PP2Cs even in the absence of ABA (Dupeux et al., 2011; Hao et al., 2011). Dimeric receptors, like PYR1 and PYL1-2, on the other hand, need ABA for their dissociation and thus have no basal activity (Dupeux et al., 2011; Hao et al., 2011). Additionally, ABA receptors are able to discriminate among the different PP2Cs and selectively inhibit them (Antoni et al., 2012; Bhaskara et al., 2012; Li et al., 2013; Tischer et al., 2017).

Despite the redundancy of PYR/PYL receptors, as evidenced by the lack of phenotypes in many single mutants (Park et al., 2009; Zhao et al., 2018), they are necessary for ABA signalling. Multiple high-order mutants result in extreme insensitivity to ABA and present severe defects in processes regulated by the hormone, such as seed germination, plant growth, stress responses or transcriptional regulation (Zhao et al., 2018). Evidence shows that PYL receptors also appear to have individual specific functions. For instance, PYL8 was shown to regulate primary root growth and, along with PYL9, is also involved in lateral root growth via interaction with MYB77 and MYB44 transcription factors to induce auxin signalling (Zhao et al., 2014; Xing et al., 2016). PYL4 and PYL5 are required for the plants responses to CO_2, while PYL2 is sufficient for guard cell responses mediated by ABA (Dittrich et al., 2019). Another receptor, PYL6, has been shown to interact with MYC2, establishing it as a potential connector between the ABA and jasmonic acid (JA) signalling (Aleman et al., 2016).

1.1.1.3 PP2Cs

Clade A PP2Cs are considered the ABA co-receptors, as they work in conjunction with the PYLs, and the major negative regulators of the ABA signalling. Although the PP2C family of phosphatases is composed of 80 members in Arabidopsis (Xue et al., 2008), only nine of them make up clade A, six of which are directly involved in the regulation of ABA responses (reviewed in Fuchs et al., 2013). They can be clustered into two different subfamilies based on sequence similarity: the ABI1 subfamily, which includes ABI1 (ABA insensitive 1), ABI2 (ABA insensitive 2), HAB1 (Homology to ABI1 1), and HAB2 (Homology to ABI1 2); and the AHG1 subfamily, containing AHG1 (ABA hypersensitive germination 1), AHG3/AtPP2CA (ABA hypersensitive germination 3/Protein phosphatase 2CA), HAI1 (Highly ABA-induced 1), HAI2 (Highly ABA-induced 2), and HAI3 (Highly ABA-induced 3) (Nishimura et al., 2018). Clade A PP2C proteins differ in their subcellular localization: the AHG1 subfamily is predominantly expressed in the nucleus, while ABI1 members are both present in the cytoplasm and nucleus (Nishimura et al., 2018). Except for HAI1-3, which are involved in ABA-independent low water potential signalling (Bhaskara et al., 2012), clade A PP2C members regulate SnRK2-activated ABA responses. Single and multiple loss-of-function mutants exhibit ABA hypersensitive phenotypes, showing decreased seed germination, plant biomass, and root growth levels upon ABA exposure, as well as enhanced stomatal closing and drought resistance (Rubio et al., 2009; Antoni et al., 2013). Although partial functional redundancy has been shown for PP2C (Saez et al., 2006; Rubio et al., 2009; Bhaskara et al., 2012), some PP2Cs have specific functions, such as AHG1 and AHG3, which are involved in ABA-regulated germination responses and whose single mutants are hypersensitive to ABA at the seed stage (Yoshida et al., 2006; Nishimura et al., 2007). PP2C phosphatases from clade A not only target SnRK2s: HAI PP2Cs also interact with MAP (mitogen-activated protein) kinases MPK3 and MPK6 for ABA-mediated immune suppression (Mine et al., 2017), and ABI1 interacts with the AIK1 MAPKKK kinase to regulate root growth and stomatal responses (Li et al., 2017a).

1.1.1.4 SnRK2s

The SnRK2 family constitutes a group of Ser/Thr kinases specific to the plant kingdom. In *Arabidopsis thaliana*, 10 members have been identified (Hrabak et al., 2003), which can be clustered into 3 different subclasses (Kulik et al., 2011). All 10 members, except for SnRK2.9, have been shown to respond to osmotic stress, while only five are activated by ABA (Boudsocq et al., 2004). In fact, the Arabidopsis decuple mutant showed severe growth defects under hyperosmotic stress conditions, but was similar to wild type plants in the absence of stress, revealing the crucial role of SnRK2s in osmotic stress responses (Fujii et al., 2011).

Members of subclass I (SnRK2.1, SnRK2.4, SnRK2.5, SnRK2.9, SnRK2.10) do not respond to ABA, members of subclass II (SnRK2.7, SnRK2.8) are weakly activated by ABA, while subclass III kinases (SnRK2.2, SnRK2.3, SnRK2.6) are strongly responsive to the hormone and are considered the central positive regulators of ABA signalling (Boudsocq et al., 2004; Kulik et al., 2011). Indeed, the *snrk2.2/snrk2.3/snrk2.6* triple mutant is fully insensitive to ABA during seed germination and the post-germination stage, demonstrating both a key function for these kinases in ABA signalling and their functional redundancy (Fujii et al., 2009; Fujita et al., 2009). At the vegetative phase, the mutant displays severe defects in ABA-mediated stress responses, with drastic downregulation of ABA-dependent regulated transcription factors (Fujii et al., 2009; Fujita et al., 2009).

Despite their functional redundancy there are differences among the subclass III kinases. SnRK2.6/OST1 (SNF1-related protein kinase 2.6/Open stomata 1) is mainly expressed in guard cells and is in charge of regulating ABA-induced stomatal movements, directly activating the SLAC1 (slow anion channel-associated 1) channel (Mustilli et al., 2002; Yoshida et al., 2002). On the other hand, SnRK2.2 and SnRK2.3 regulate ABA signalling during seed germination and seedling growth (Fujii et al., 2007).

SnRK2s can be activated in an ABA-dependent (PYL-PP2C) or ABA-independent (B Raf-like kinases) manner. Until recently, subclass III SnRK2s were assumed to become active through ABA-induced autophosphorylation events at specific Ser residues. However, recent studies in Arabidopsis have shown ABA-independent activation of SnRK2s in response to osmotic stress via upstream B2 and B3 Raf-like kinases, a pre-requisite for the re-activation of SnRK2s from their PP2C-mediated inhibition and crucial for ABA signalling (Katsuta et al., 2020; Lin et al., 2020; Takahashi et al., 2020). Interestingly, ABA-independent SnRK2 activation via osmotic stress was enhanced in the PYL duodecuple mutant, which is fully blocked in ABA-dependent signalling, suggesting that ABA-dependent SnRK2 activity antagonizes ABA-independent regulation, perhaps as a compensatory mechanism (Zhao et al., 2018). SnRK2s have also been recently shown to regulate the growth regulator target of rapamycin (TOR), integrating plant growth and stress responses (Wang et al., 2018c; Belda-Palazón et al., 2020).

1.1.1.5 **ABA effectors**

Upon activation, SnRK2s phosphorylate numerous downstream targets, including transcription factors and ion channels, subsequently leading to protective stress responses (Umezawa et al., 2013; Wang et al., 2013). Studies have reported that 4-30% of the Arabidopsis genome is altered in expression by ABA (Nemhauser et al., 2006; Choudhury and Lahiri, 2011; Yang et al., 2014; Costa et al., 2015; Song et al., 2016b; Sun et al., 2022), with percentages depending on factors such as the duration and concentration of the treatment, age of seedlings and tissues analysed, as well as the data processing methods. This suggests that, under the right conditions, a significantly higher ABA-responsiveness could be achieved. As indicated by Nemhauser et al., around 12% of the ABA-responsive genes are associated with transcription and primarily include transcription factors from the bZIP (basic leucine zipper-type protein), AP2/ERF (APETALA2/ethylene responsive binding factor), MYB (myeloblastosis), MYC (myelocytomatosis), NAC (NAM [no apical meristem], ATAF [Arabidopsis transcription activation factor], CUC [cup-shaped cotyledon]), and WRKY (WRKY DNA-binding protein) families, among many others, which act as repressors or activators depending on the target. Previous studies had also identified the bZIP transcription factor subfamily of AREB/ABFs (ABA-responsive element [ABRE] binding proteins/ABRE-binding factors), which bind to ABRE *cis* elements in ABA-responsive genes to modulate their transcription (Fujita et al., 2005; Furihata et al., 2006; Yoshida et al., 2010; Yoshida et al., 2015). Indeed, the *areb1/abf2 areb2/abf4 abf3 abf1* quadruple mutant showed ABA insensitivity, indicating they are the main transcription factors for ABA-dependent responses (Yoshida et al., 2010; Yoshida et al., 2015). ABI5 (ABA-insensitive 5) is another key member of the bZIP family of transcription factors activated by ABA that plays a major role in growth inhibition under stress. The *abi5* loss-of-function mutant is ABA insensitive compared to the wild type during seed germination and exhibits lower tolerance to salt stress (Finkelstein, 1994; Chang et al., 2019). Both AREB/ABFs and ABI5 have been shown to regulate gene expression during the vegetative growth stage as well as at the seed germination level, and can act synergistically (Lynch et al., 2012; reviewed in Collin et al., 2021). They have also been shown to participate in the attenuation of ABA signalling via activation of PP2Cs (Lynch et al., 2012; Wang et al., 2019a). Another transcription factor involved in ABA signalling is WRKY40 which functions as a suppressor of ABA responses under control conditions by binding to the promoters of AREB2/ABF4 or ABI5 (Shang et al., 2010). The activity of ABA effectors can also be negatively regulated at the post-translational level. Examples include the dephosphorylation of ABI5 by PP6 (protein phosphatase 6) (Dai et al., 2013), the inactivation of RAV1 (related to ABI3/VP1 1), a negative regulator of ABA signalling, through SnRK2s phosphorylation (Feng et al., 2014), and the degradation of ABI5 by the KEG (keep on going) E3 ligase (Liu and Stone, 2013). At the seed stage, the B3-domain transcription factor ABI3 (abscisic acid insensitive 3) plays a crucial role in seed maturation and germination, positively regulating ABA signalling (Finkelstein et al., 2008; Sano et al., 2016).

In 2013, two parallel phosphoproteomic studies conducted on the Arabidopsis *snrk2.2/2.3/2.6* triple mutant were able to confirm direct interaction of SnRK2s with AREBs and identify new ABA-activated substrates (Umezawa et al., 2013; Wang et al., 2013). In addition to AREB/ABFs, proteins involved in a myriad of cellular processes were also identified as SnRK2 substrates, such as DNA- and RNA-binding proteins, including splicing factors, flowering regulators or proteins involved in chloroplast processes or epigenetics (Umezawa et al., 2013;

Wang et al., 2013). SNS1 (SnRK2-substrate 1) is one of the SnRK2 targets identified and was found to function as a negative regulator of ABA signalling, with its knockout mutants being hypersensitive to ABA (Umezawa et al., 2013). Other examples of SnRK2 targets, specifically of SnRK2.6/OST1, include ion channels such as SLAC1, KUP6 (K^+ uptake transporter 6) and KAT1 (K^+ channel in Arabidopsis thaliana 1), responsible for ABA-mediated stomatal closure (Geiger et al., 2009; Sato et al., 2009; Osakabe et al., 2013), the NADPH oxidase RbohF (Respiratory burst oxidase homolog protein F), involved in ABA-induced reactive oxygen species (ROS) production (Sirichandra et al., 2009), or the epigenetic-related SWI/SNF chromatin-remodelling ATPase BRM (BRAHMA), which prevents the activation of ABA responses in the absence of stress (Peirats-Llobet et al., 2016).

ABA mediates plant responses to conditions that induce osmotic stress, such as drought or high salinity. It is therefore not surprising that approximately ~25-50% of ABA-regulated genes are shared by the plant's responses to drought or salt stress (reviewed in Finkelstein, 2013). The remaining response is led by ABA-independent pathways specific to each type of stress and regulated by distinct transcription factors, such as the DREB (Drought response element binding) protein family which contains members functionally associated to drought, but also salinity and cold regulation (reviewed in Finkelstein, 2013).

1.1.1.6 Regulation of the ABA pathway

For plants to adequately respond to the severity of the stress perceived and prevent overreaction, as well as to suppress stress signals under normal conditions to promote growth, the ABA signalling pathway requires both positive and negative regulators. These regulators must modulate the intensity of the signal by terminating it or desensitizing the pathway in response to sustained stimuli. They must also be able to properly coordinate the different regulatory processes, as the core ABA signalling components are tightly regulated at the transcriptional, post-transcriptional or post-translational levels, and through protein-protein interactions.

1.1.1.6.1 Transcriptional regulation

At the transcription level, the expression of *PP2Cs* is well known to be upregulated by ABA through AREB/ABFs to reduce the signalling and maintain an equilibrium (Wang et al., 2019a), while *PYLs* have been shown to be both upregulated and downregulated upon exogenous ABA, depending on the gene and on whether the signal is being amplified or attenuated (Bhaskara et al., 2012; Li et al., 2018a; Dittrich et al., 2019; Zhao et al., 2020). On the contrary, very little is known regarding the transcriptional regulation of SnRK2s. In some crop species, upregulation of SnRK2s has been shown depending on the plant species and type of stress, but this has been suggested to be a feedback response to the degradation of the protein in order to fine-tune the stress response (reviewed in Maszkowska et al., 2021). LHY (late elongated hypocotyl), a circadian regulator, has been shown to directly interact with SnRK2s as well as other components of the ABA signalling and metabolic pathways, altering their expression levels to enhance or suppress ABA responses depending on the time of day and stage of development (Adams et al., 2018).

1.1.1.6.2 Post-translational regulation

Post-translational modifications, mainly phosphorylation/dephosphorylation and ubiquitination, play an important role in the functional regulation and stability of ABA signalling components both under normal and stress conditions. Degradation of proteins from the ABA signalling pathway via the ubiquitin-26S proteasome system along with the endocytic/vacuolar degradation pathway provides a means of attenuating ABA responses (reviewed in Coego et al., 2021). Degradation of ABA receptors and SnRK2s contributes to desensitization of the pathway, as evidenced by the reduction in ABA sensitivity upon overexpression of E3 ligases (reviewed in Lim et al., 2022). Plant U-box 22 and 23 (PUB22/23), DDA1 (DET1-,DDB1-ASSOCIATED 1), RSL1 (RING finger of seed longevity 1), VPS23A (vacuolar protein sorting 23A), and ALIX (ALG-2 interacting protein-X) regulate PYL degradation, and plants overexpressing these proteins show reduced sensitivity to ABA (Bueso et al., 2014; Irigoyen et al., 2014; Yu et al., 2016; Zhao et al., 2017; García-León et al., 2019). On the other hand, HOS15 (high expression of osmotically responsive genes 15) and PP2-B11 (phloem protein 2-B11) promote SnRK2s degradation (Cheng et al., 2017; Ali et al., 2019). However, degradation of PP2Cs leads to both activation and resetting of ABA signalling, of special importance when the stress ceases to exist. The presence of ABA promotes the degradation of ABI1 and PP2CA by E3 ubiquitin ligases, PUB12/PUB13 (plant U-box 12/13) (Kong et al., 2015), or RGLG1/RGLG5 (RING [really interesting new gene] domain ligase1/5) (Wu et al., 2016) and PIR1/PIR2 (PP2CA-interacting RING finger protein 1/2) (Baek et al., 2019b), respectively.

Another major post-translational modification is phosphorylation, catalysed by protein kinases and reversed by phosphatases, which can affect the target's activity, stability, localization, and interaction with other proteins. ABA receptors PYL8 and PYR1 are stabilized by CARK1 (Cytosolic ABA receptor kinase 1) phosphorylation, enhancing its ability to inhibit ABI1 (Zhang et al., 2018). On the contrary, AEL (Arabidopsis EL1[Early flowering 1]-like), TOR, and CEPR2 (C-terminally encoded peptide receptor 2) phosphorylate PYLs to suppress ABA signalling in the absence of stress and prevent ABA-mediated growth inhibition (Chen et al., 2018a; Wang et al., 2018c; Yu et al., 2019). PP2Cs and SnRK2s can also undergo positive or negative regulation via phosphorylation. SnRK2s can be phosphorylated and activated by Raf-like kinases (reviewed in Ma et al., 2022), BIN2 (Brassinosteroid insensitive 2) (Cai et al., 2014), BAK1 (BRI1 [Brassinosteroid-insensitive 1]-associated receptor kinase 1) (Shang et al., 2016), and RPK1 (receptor-like protein kinase 1) (Shang et al., 2020). Conversely, in maize, ZmOST1 is phosphorylated by CK2 (casein kinase 2), enhancing its interaction with PP2Cs, thereby inactivating it or triggering its proteasomal degradation (Vilela et al., 2015). On the other hand, besides PP2Cs inactivation of SnRK2s, TOPP1 (type one protein phosphatase 1) inhibits OST1 kinase activity via dephosphorylation (Hou et al., 2016). As for the PP2Cs, PR5K2 (PR5 receptor-like kinase 2) phosphorylates ABI1 and ABI2 and represses ABA signalling (Baek et al., 2019a). Recently, Li and colleagues identified a positive role for EGR1/2 (clade E growth-regulating 1 and 2) phosphatases in SnRK2.2 activity, enhancing ABA responses (Li et al., 2023).

1.1.1.6.3 Protein-protein interactions

Aside from post-translational modifications, at the protein level, the ABA pathway components can also be regulated via protein-protein interactions. EAR1 (Enhancer of ABA co-receptor 1) interacts with PP2Cs, enhancing their activity and negatively affecting the ABA response (Wang et al., 2018a), or ROP11 (Rho-related protein from plants 11), which interacts with ABI1 and ABI2, protecting their phosphatase activity from PYL inhibition (Li et al., 2012; Yu et al., 2012). Recently, ABT (ABA signalling terminator) has been shown to reduce ABA signalling by blocking the interaction between PYLs and PP2Cs (Wang et al., 2020). By contrast, RDK1 (receptor dead kinase 1) recruits ABI1 to the plasma membrane, positively regulating ABA responses (Kumar et al., 2017).

1.1.1.6.4 Post-transcriptional regulation

Post-transcriptional regulation of gene expression controls the amounts of mRNAs (messenger RNAs) available for translation, ultimately affecting protein levels. It involves processes such as alternative splicing, RNA editing, or RNA silencing. Alternative splicing is a major regulatory mechanism that, through combinations of different exonic sequences, results in the production of multiple transcripts from a single pre-mRNA (precursor-mRNA) molecule and has been shown to play an important role in stress responses (reviewed in Staiger and Brown, 2013; Cui and Xiong, 2015; Filichkin et al., 2015; Laloum et al., 2018; Punzo et al., 2020a). The core components of ABA signalling also undergo regulation at the splicing level – see section 1.2.3.

1.1.2 ABA homeostasis: biosynthesis, catabolism, and transport

ABA plays crucial functions in plant physiology, development, and stress responses. To carry out these functions properly, plants must regulate their endogenous ABA levels which are a net result of its production, catabolism, and activation/inactivation. The compartmentalization of the hormone and its transport throughout the plant are also key aspects in the ABA metabolic network. ABA metabolism, summarized in Figure 1.2, is regulated not only by ABA itself, but also by developmental and environmental cues. The interconnectedness of ABA with other hormonal pathways, such as auxin, adds another layer of complexity to its homeostasis.

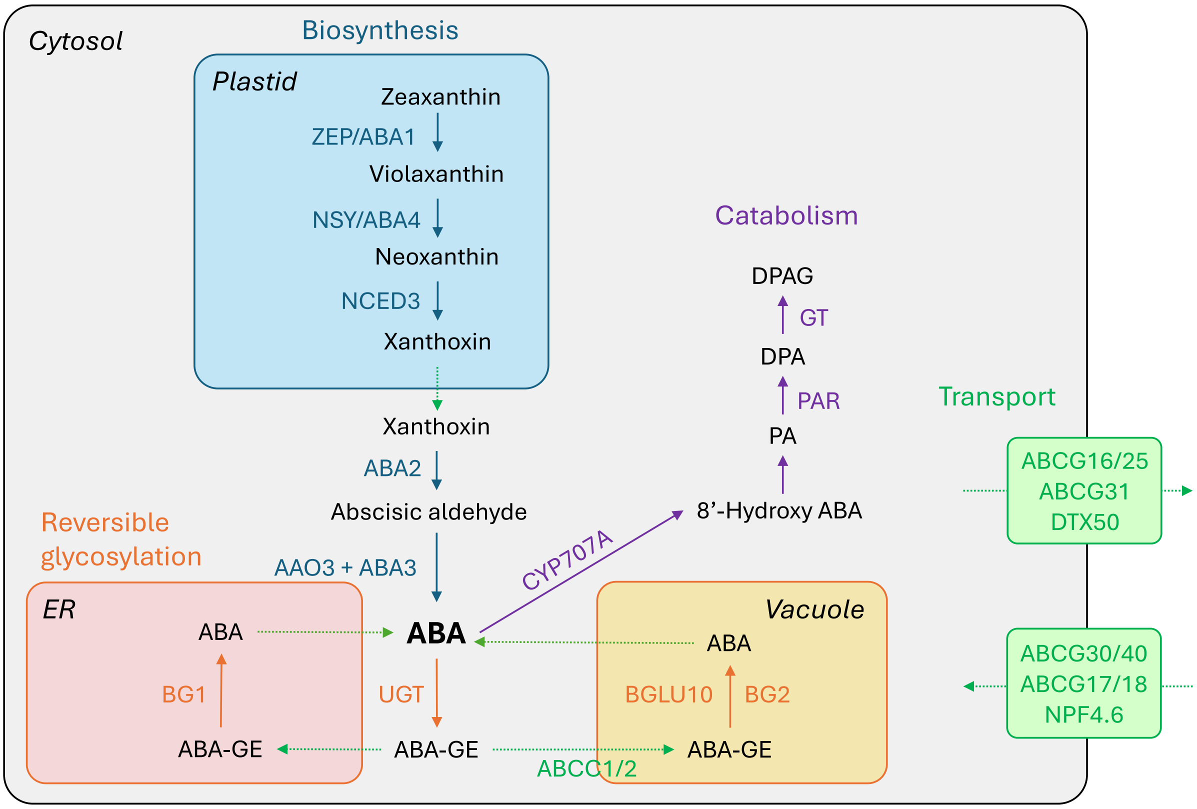

Figure 1.2. Overview of ABA metabolism and transport.

ABA biosynthesis (shown in blue) begins in the plastid with the conversion of zeaxanthin into xanthoxin, which is then exported to the cytosol to be converted into ABA. ABA production is balanced by its catabolism (shown in purple) and reversible glycosylation (shown in orange) pathways. ABA export and import transport routes are marked with green and dashed lines. Figure adapted from Díez et al., 2024.

1.1.2.1 Biosynthesis

ABA biosynthesis is crucial not only for basal activities but also for the required rapid accumulation of the hormone in response to stress. Although ABA is present in a wide range of organisms, its production differs. ABA is produced from carotenoid intermediates via a multistep pathway that takes place across different cellular compartments and involves multiple enzymes (reviewed in Nambara and Marion-Poll, 2005; Finkelstein, 2013). First, carotenoid precursors are synthesized in plastids via the methylerythritol phosphate (MEP) pathway. Subsequent reactions lead to the production of carotenoids, out of which only β-carotene will be used for ABA biosynthesis upon conversion to zeaxanthin. There are three main steps in ABA production inside the plastids: 1) zeaxanthin is converted to violaxanthin via a two-step reaction catalysed by zeaxanthin epoxidase/ABA-deficient 1 (ZEP/ABA1); 2) violaxanthin can be converted into neoxanthin via neoxanthin synthase/ABA-deficient 4 (NSY/ABA4), and both violaxanthin and neoxanthin are transformed via unknown enzymes into 9-*cis*-violaxanthin and 9’-*cis*-neoxanthin, respectively; 3) these carotenoids are cleaved by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) to produce xanthoxin, which is exported to the cytosol for the final steps of ABA production. This is considered a rate-limiting step in ABA biosynthesis, and as such, the expression of *NCED* is under tight regulation and correlates with endogenous ABA levels. Loss of function mutants of plastidial steps in ABA biosynthesis do not completely lack ABA, thus, recently, an alternative cytosolic pathway has been proposed by Jia et al. (2021), independent of ZEP/ABA1, in which the production of xanthoxin comes from carotenoids produced from β-carotene or zeaxanthin. The cytosolic steps of ABA production involve the enzyme ABA-deficient 2 (ABA2), which converts xanthoxin to abscisic aldehyde, and a final step to produce ABA catalysed by abscisic aldehyde oxidase 3 (AAO3), which requires a molybdenum cofactor (MoCo) for its function that is produced by ABA-deficient 3 (ABA3). Although it was previously assumed that roots were the main source of ABA (Zhang et al., 1987), research has revealed that ABA is mainly derived from leaves and transported to the roots to maintain ABA levels and normal growth in that tissue (Manzi et al., 2015; McAdam et al., 2016).

1.1.2.2 Catabolism

In order to regulate the amount of ABA, its synthesis must be balanced by its catabolism. There are two major ABA catabolism mechanisms: irreversible hydroxylation and reversible conjugation. On one hand, ABA can be sequentially hydroxylated yielding predominantly 8’-OH ABA, a reaction catalysed by the CYP707A family of cytochrome P450 monooxygenases consisting on 4 members (CYP707As 1, 2, 3 and 4), with varying spatial and temporal expression patterns (Krochko et al., 1998; Kushiro et al., 2004; Saito et al., 2004). 8’-OH ABA is spontaneously isomerized to phaseic acid (PA), which is finally converted to dihydrophaseic acid (DPA) and DPA-4-O-β-D-glucoside (DPAG) by PA reductase ([PAR], encoded by *ABA hypersensitive 2 [ABH2]*) and glycosyltransferase (GT), respectively (Weng et al., 2016). Notably, both 8’-OH ABA and PA exhibit some ABA-like activity, although to a significantly lower extent than ABA itself (Kepka et al., 2011; Weng et al., 2016).

On the other hand, ABA or its metabolites can undergo inactivation through conjugation with glucose, a process catalysed by UDP-glucosyltransferase (UGT) enzymes, with ABA glucosyl ester (ABA-GE) being the major conjugate. Various members of the UGT superfamily in Arabidopsis have been described to possess ABA catalytic activity. UTG71C5 is considered the key enzyme in sugar conjugation to ABA, as knockout plants exhibit ABA-excessive phenotypes, with increased induction of ABA-responsive genes (Liu et al., 2015). Also proven to have in vivo ABA glucosyltransferase activity are UGT71B6 and its two homologues, UTG71B7 and UTG71B8, which when co-suppressed, plants become hypersensitive to ABA (Dong et al., 2014).

Unlike the hydroxylation pathway, conjugation is reversible through the hydrolysis of ABA-GE by β-glucosidases, resulting in free bioactive ABA (Lee et al., 2006; Xu et al., 2012). This process occurs in two different compartments and involves different genes. BG1 is located in the endoplasmic reticulum (ER) and loss-of-function mutants are severely ABA-deficient (Lee et al., 2006). On the other hand, two β-glucosidases, BG2 and BGLU10, were found in the vacuole. Owing to existing functional redundancy, loss-of-function mutants of *BG2* and *BGLU10* only exhibit a mild phenotype when compared to *BG1* (Wang et al., 2011; Xu et al., 2012). Although these three genes are induced in response to stress, their products differ in activation mechanism: BG1 undergoes stress-induced polymerization (Lee et al., 2006; Watanabe et al., 2014), BG2 exists as stable polymers under normal conditions and remains protected from degradation during stress (Xu et al., 2012), whereas the activation mechanism of BGLU10 is yet to be studied.

Compared to the multistep pathway of de novo ABA biosynthesis, the hydrolysis of ABA-GE represents a quick and efficient one-step process, enabling plants to rapidly increase ABA levels. ABA-GE serves as a storage or transport form of ABA, accumulating in vacuoles and the apoplast (Jiang and Hartung, 2008; Burla et al., 2013). During stress conditions, ABA-GE undergoes hydrolysis, either from the stored ABA-GE in the vacuole or from ABA-GE transported into the ER for this purpose, thereby facilitating the conversion to active ABA to initiate signalling in response to stress (Lee et al., 2006; Wang et al., 2011; Han et al., 2020).

1.1.2.3 Regulation of ABA metabolism

When plants face stress, both ABA biosynthesis and catabolism are concurrently activated. This requires a balance between the pathways to maintain the optimal hormone levels that suit the plant's needs. The regulation of ABA metabolic pathways involves a multifaceted interplay of diverse regulatory mechanisms. These include the modulation of gene expression and stability by transcription factors, microRNAs, or mRNA decapping proteins, as well as the influence of chemicals and signalling peptides. The mRNA levels of *NCED3*, the key ABA biosynthetic enzyme, and *CYP707As*, responsible for ABA inactivation, are known to correlate with plant ABA levels. This correlation indicates that their transcriptional regulation plays a crucial role in orchestrating a proper response to stress (Kushiro et al., 2004). The *NCED3* promoter is subjected to the binding of numerous transcription factors, with some, like ATAF1 or NGATHA1 (NGA1) playing a positive role in drought responses (Jensen et al., 2013; Sato et al., 2018). Conversely, homeodomain-leucine zipper proteins 1 (HAT1) and 3 (HAT3) negatively regulate *NCED3*, as well as *ABA3*, under drought stress conditions (Tan et al., 2018). Additionally, the mRNA turnover of *NCED3* is influenced by SM-like proteins 1-7 (LSM1-7) from an mRNA decapping complex under low temperatures (Perea-Resa et al., 2016). Recently, the root-derived CLE25 (CLAVATA3/Embryo surrounding region-related 25) peptide has emerged as a regulator of the ABA signalling pathway, binding to BAM (barely any meristem) receptors and promoting ABA biosynthesis through upregulation of *NCED3* expression (Takahashi et al., 2018). Furthermore, sulfate has been identified as a chemical signal to induce *NCED3* expression in guard cells to promote stomatal closure during drought conditions (Malcheska et al., 2017).

NCED3 transcript levels are also directly regulated by ABA through an ABRE in its promoter (Yang and Tan, 2014). Similarly, *CYP707As*, involved in ABA catabolism, are subjected to transcriptional regulation by multiple genes during drought conditions, being induced by VIP1 (VirE2-interacting protein 1) and repressed by bHLH122 (basic helix-loop-helix 122) or SVP (short vegetative phase) (Tsugama et al., 2012; Liu et al., 2014; Wang et al., 2018e). Moreover, the flowering repressor SVP contributes to improved drought tolerance by inducing *BG1* expression, and miR165/166 also modulates *BG1* transcript levels (Yan et al., 2016). These findings, though only representing a fraction of the intricate regulatory mechanisms in action, show the complexity of the regulatory network used by plants to finely tune ABA metabolism in response to stress.

1.1.2.4 Transport

To achieve ABA homeostasis, plants must not only coordinate and balance its production and inactivation, but also its transport across the plant. ABA is primarily produced in the vasculature and its transport across cells and tissues is crucial to carry out its functions in growth, development and stress responses.

ABA is a weak acid, that primarily exists in an ionized form which makes it difficult for the hormone to freely diffuse across membranes (reviewed in Boursiac et al., 2013). Thus, transporter proteins are necessary for active ABA translocation to its target sites. ABA transporters that move the hormone and/or its conjugates across membranes include members from the ATP-binding cassette (ABC) class G and class C, nitrate transporter 1/peptide transporter family (NPF), and detoxification efflux carriers (DTX)/multidrug and toxic compound extrusion (MATE) families of transporters (see Figure 1.2).

ABCG40 and ABCG30 import ABA into guard cells and the embryo (Kang et al., 2010; Kang et al., 2015). Recently, ABCG17 and ABCG18 were described to transport ABA into shoot mesophyll and cortex cells, promoting ABA-GE formation in these sink tissues and being repressed under abiotic stress conditions (Zhang et al., 2021b). NPF4.6 also imports ABA into the vasculature to regulate stomatal aperture (Kanno et al., 2012). On the contrary, ABCG25 and ABCG31 transport ABA out of the vasculature, endosperm and guard cells (Kuromori et al., 2010; Kang et al., 2015). DTX50, from the MATE family, also exports ABA out of vasculature and guard cells (Zhang et al., 2014). Two members of class C of ABC transporters located in the tonoplast, ABCC1 and ABCC2, are in charge of importing ABA-GE into the vacuole along with the action of antiporters, for storage and its posterior activation according to the plant's needs (Burla et al., 2013). A recent report describes a new function for ABCG25, along with ABCG16. Under stress conditions, both transporters act as homodimers in the plasma membrane, exporting ABA to the apoplast, however, its basal activity during early plant development occurs as heterodimers in the ER membrane, shipping ABA-GE to the inside of the organelle for its activation by *BG1* (Zhou et al., 2024).

Both ABA and ABA-GE undergo translocation between roots and shoots via the plant vasculature. Long-distance root-to-shoot transport of ABA and ABA-GE, facilitated by ABCG25, is essential to regulate stomatal aperture specifically under non-stress conditions (Yang et al., 2024). Conversely, during drought stress, shoot-derived ABA, induced by CLE25 from the roots, is key for stomatal closure (Takahashi et al., 2018). Moreover, abiotic stress reduces the expression of ABCG17 and ABCG18 transporters, resulting in the translocation of foliage-derived ABA to the roots to regulate lateral root growth (Zhang et al., 2021b).

1.1.3 Functions of ABA

ABA is a polyvalent molecule, whose functions in regulating plant growth, development and responses to stress have been extensively reviewed (reviewed in Finkelstein, 2013; Ma et al., 2018; Yoshida et al., 2019; Chen et al., 2020; Ali et al., 2022). ABA functions in the plant start as early on as the seed stage, where it controls maturation, dormancy, and finally germination. During the seedling and adult plant phases, ABA regulates root architecture, shoot growth, stomatal development and transpiration, as well as the floral transition and senescence. These are all functions that the plant, with the help of ABA and other phytohormones, will modify accordingly upon the encounter of unfavourable conditions, either biotic or abiotic.

1.1.3.1 ABA functions in development

ABA is well known to regulate various developmental processes throughout the plant's life cycle, from seed maturation to senescence. The seed progresses through distinct phases: maturation, dormancy, and germination. During maturation, an initial peak of maternally-derived ABA arrests cell division, prompting the embryo to initiate the accumulation of storage reserves by activating transcription factors like ABI3, FUSCA3 (FUS3), leafy cotyledon 1/2 (LEC1/2), and LEC1-like (L1L), composing the LAFL network (Raz et al., 2001; Finkelstein et al., 2002; Jia et al., 2014). These factors facilitate de novo ABA biosynthesis, inducing the synthesis of storage proteins (reviewed in Jia et al., 2014). Later on, a second peak of ABA levels and the action of key genes like ABI4 and ABI5 are crucial to confer desiccation tolerance and induce seed dormancy to protect the embryo integrity, through accumulation of sugars, antioxidants and late embryogenesis abundant (LEA) proteins, and prevent premature germination until the right conditions are met (Finkelstein, 1994; reviewed in Finkelstein et al., 2002; Shu et al., 2013). The release of seed dormancy and initiation of germination are intricately regulated by ABA catabolism and the balance between ABA and Gibberellic acid (GA) (reviewed in Vishal and Kumar, 2018). During seed dormancy, elevated ABA levels are maintained through the induction of its biosynthetic genes by transcription factors like MYB96 and ABI4, which also suppress GA biosynthesis (Lee et al., 2015; Shu et al., 2016). In Arabidopsis, the dormancy release can be triggered by cold temperatures and exposure to light and is marked by a rapid decline in ABA levels caused by CYP707A2, coupled with an increase in GA levels that facilitates seedling emergence (Yamauchi et al., 2004; Okamoto et al., 2006; Weitbrecht et al., 2011; Lee et al., 2015). If the conditions are favourable, the embryonic root or radicle will emerge from the seed coat, after testa and endosperm rupture facilitated by the expansion of hypocotyl cells (Penfield, 2017). On the other hand, upon stress ABA will suppress germination by inducing dormancy. During early growth and until the photoautotrophic status is achieved, all the mechanisms and energy consumption are supported by the stored reserves acquired during seed maturation (Penfield, 2017). The transition to becoming a photosynthetic, self-sustaining organism implies the establishment of a functional root system, the development of chloroplasts, and the greening of cotyledons. However, if the conditions are unfavourable, seedlings undergo a post-germination growth arrest as a survival adaptive strategy (Lopez-Molina et al., 2001). This process is mediated by ABA, as ABA-deficient or ABA-insensitive mutants are unable to arrest growth (Barrero et al., 2005). ABI5, in addition to regulating desiccation tolerance and germination, also plays a major role in this stress-induced growth inhibition at the post-germination stage (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002) and is an integration point between ABA signalling and other hormones and external cues (Yadukrishnan et al., 2020). The ABA-induced post-germination growth arrest is reversible upon removal of stress conditions (Lopez-Molina et al., 2001).

One of the crucial events during early seedling development is the establishment of photosynthetic activity. For such, the primordial leaves or cotyledons develop chloroplasts and accumulate chlorophyll, turning green and expanding upon light exposure (reviewed in Yadukrishnan and Datta, 2021). This process is regulated by another major phytohormone, cytokinin (CK), which antagonizes the ABA-mediated inhibition by promoting the proteasomal degradation of ABI5 (Guan et al., 2014). ABA also regulates leaf and shoot growth via inhibition of ethylene production (Sharp et al., 2000), and negatively regulates hypocotyl elongation in the dark by affecting GA metabolism, stabilizing DELLA proteins and repressing auxin biosynthetic genes (Lorrai et al., 2018). Moreover, the development of stomata, which are pores in the epidermis of terrestrial plants key for controlling transpiration and CO_2 uptake, is inhibited by ABA (Tanaka et al., 2013). Stomatal movements, covered in section 1.1.3.3, are also ABA regulated, controlling transpiration rates and promoting closure of stomata under adverse conditions to prevent water loss or pathogen entry (reviewed in Bharath et al., 2021).

As in the seed, ABA plays a dual role, inhibition and promotion, in the developmental regulation of roots, the underground structures in charge of anchoring the plant in the soil, absorbing water and nutrients, as well as storing food reserves. Primary roots (PRs) require ABA for their normal growth, as substantiated by the shorter PR of the ABA-deficient mutant *aba2*, which restores normal growth upon exogenous ABA (Lin et al., 2007). The effect of ABA on PR is dosage-dependent. Low concentrations stimulate PR growth via auxin signalling and efflux through PIN2/EIR1, while high ABA concentrations inhibit growth through an ethylene-dependent pathway requiring auxin signalling and influx through AUX1 (Li et al., 2017b). Additionally, ABA regulates hydrotropism, a differential root growth response to asymmetric water availability, via the action of SnRK2.2 (Dietrich et al., 2017). On the other hand, ABA impacts lateral root (LR) development, which in Arabidopsis initiates post-embryonically. While auxin is the major regulator, ABA also modulates LR growth, as evidenced by loss-of-function mutants of ABA biosynthetic and signalling genes presenting altered LR numbers (Cheng et al., 2002; Deak and Malamy, 2005; Shkolnik-Inbar and Bar-Zvi, 2010). The effect of ABA on LR growth depends on the stage of development. LR formation has several phases: initiation, primordium development, emergence, meristem activation, and elongation (reviewed in Harris, 2015). In Arabidopsis, ABA exerts opposite roles during LR primordia (LRP) formation, with ABI3 inducing this process and ABI4 having the opposite effect, both working through auxin but acting on different pathways (Brady et al., 2003; Shkolnik-Inbar and Bar-Zvi, 2010). Additionally, ABI4 represses LR elongation (Shkolnik-Inbar and Bar-Zvi, 2010). Exogenous ABA treatment stalls meristem activation post-emergence (De Smet et al., 2003), but upon prolonged exposure, during the recovery phase, PYL8 and PYL9 promote LR elongation after ABA inhibition through MYB44 and MYB77 transcription factors, connecting ABA to auxin independently from the PP2C-SnRK2 axis (Zhao et al., 2014; Xing et al., 2016). Furthermore, ABA transporters play an important role in LR development. In the absence of stress, ABCG17 and ABCG18 restrict translocation of ABA from the shoot to the root, whereas under abiotic stress conditions, their repression inhibits LR elongation (Zhang et al., 2021b).

ABA is also known to regulate the floral transition, from the vegetative to the reproductive growth stages, with both positive and negative roles on flowering time (reviewed in Conti, 2017).

Senescence marks the final phase of plant development and can be the result of aging or occur prematurely in response to stress as a survival strategy. During senescence, resources are redistributed from source senescing tissues to sink organs and meristems. ABA promotes senescence by activating the transcriptional regulators that induce the expression of senescence-associated genes (reviewed in Asad et al., 2019; Hewage et al., 2020).

1.1.3.2 Role in stress responses

Despite its important roles in plant development, ABA is best known as a major stress response hormone. The exposure to exogenous ABA or abiotic stress conditions, such as drought, high salinity, or extreme temperatures, cause an osmotic imbalance in the plant and leads to the accumulation of ABA. This triggers two types of ABA-signalling responses in the plant: short- and long-term. To cope with the immediate effects of the stress, plants respond by closing stomata – leaf epidermal pores surrounded by two guard cells – to control transpiration rates, water loss, and prevent pathogen entry, and by activating stress-responsive genes. The stomatal response, primarily based on signalling cascades rather than transcription, involves ABA core signalling components and ion channels, and takes place within a few minutes (Huang et al., 2019b). The fastest way for a plant to obtain active ABA is through the one-step hydrolysis of stored ABA-GE by the β-glucosidases in the ER and vacuole (Lee et al., 2006; Xu et al., 2012). However, guard cells also obtain ABA from autonomous biosynthesis, from ABA transported from the roots or from ABA produced in the vasculature and then transported to guard cells (reviewed in Hewage et al., 2020). Longer-term responses are involved in adaptation to stress or stress tolerance and include the induction of seed dormancy or the regulation of shoot and root growth (reviewed in Hewage et al., 2020). Massive changes in gene expression underlie the adaptive physiological responses. Environmental stress triggers the expression of ABFs through ABA-dependent phosphorylation via its core signalling pathway (reviewed in Fujita et al., 2011). Interestingly, cold, drought, and high salinity conditions share many common targets with exogenous ABA treatment, supporting the role of ABA as a central integrator of abiotic stress responses (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2006; Zeller et al., 2009). Early transient ABA-induced changes, peaking at ~3 hours, involve genes encoding TFs, protein kinases and phosphatases, and early stress responsive genes, such as ERD (early response to dehydration) genes (reviewed in Finkelstein, 2013; Hewage et al., 2020). Late-responsive genes are in charge of adaptive tolerance responses and include responsive to desiccation (RD), cold-responsive (COR), LTI (Low temperature-induced) and KIN (cold-inducible) genes, encoding proteases, chaperonins, solute-metabolism enzymes, ion and water channels or ROS-detoxifying enzymes (reviewed in Finkelstein, 2013; Hewage et al., 2020).

ABA responses follow a temporal pattern dependent on the duration of the exposure to the hormone. Transcriptionally, genes involved in drought stress responses, ABA biosynthesis, chloroplast development and chlorophyll degradation were highly induced in the early time points after ABA treatment and less induced or repressed later on, suggesting plants tailor their responses to their duration of the stress, changing from maintaining cellular activity to a survival mode of minimal functions under prolonged stress conditions (Wang et al., 2018b). During drought, salt or cold stress, ABA helps the plant deal with ionic and oxidative imbalances, as well as osmotic stress. Apart from stomatal closure, where ABA-induced ROS might serve as a signal, ABA induces the production of osmoprotectant compounds, such as proline, antifreeze proteins, and mediates the upregulation of antioxidant enzymes involved in ROS scavenging (reviewed in Hewage et al., 2020).

Although ABA is considered the main abiotic stress hormone, it has also been connected to plant immunity. Defence responses are mainly carried out by other hormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), which are interconnected to ABA. Some ways ABA helps plants deal with biotic stressors include promoting stomatal closure to block pathogen entry and inducing callose accumulation to restrict pathogen spread (Oide et al., 2013; Melotto et al., 2017). However, ABA signalling can be highjacked by pathogens and render the plants susceptible to infection, as high ABA levels are known to inhibit the expression of resistance genes (Hu et al., 2022; Roussin-Léveillée et al., 2022). ABA also plays a role in heavy metal and UV light stress by helping in the scavenging of the overproduced ROS, and participates in the submergence-mediated hypoxia stress responses downstream of ET (reviewed in Hewage et al., 2020).

1.1.3.3 ABA regulation of stomatal aperture

Stomata are openings in the plant leaf epidermis crucial for the regulation of gas exchange during photosynthesis and for controlling water loss via transpiration, of special importance under challenging conditions, as well as an entry point for plant pathogens (reviewed in Munemasa et al., 2015). Stomatal pores are surrounded by two differentiated guard cells that regulate the opening of the pore through osmotic stress-mediated changes in turgor pressure (reviewed in Munemasa et al., 2015). Regulation of stomatal aperture is a rapid response to various environmental stimuli, such as light, CO_2, pathogens, drought, or ABA, which can take up to a few minutes and involves the ABA core signalling pathway, and ion channels at the plasma membrane of guard cells (reviewed in Munemasa et al., 2015) (see Figure 1.3). Upon encountering stress, ABA levels increase in guard cells, either imported from the vasculature from surrounding tissues, transported from the roots, or synthesized in the guard cells themselves for a rapid response (Endo et al., 2008; Bauer et al., 2013; Yang et al., 2024), activating the core ABA signalling pathway. In the presence of ABA, the release of Ca^{2+} ion channels from PP2C inhibition facilitate the import of Ca^{2+}, increasing its intracellular concentration (Brandt et al., 2015). This surge in calcium triggers the activation of calcium-dependent protein kinases (CPKs), which subsequently phosphorylate and activate anion efflux channels (Brandt et al., 2015). Moreover, the ABA-mediated inhibition of proton pumps that serve as the driving force for the activity of anion transporters results in depolarization of the plasma membrane and reduction of guard cell turgor pressure, culminating with the closure of the stomatal pore (Brault et al., 2004). The control of stomatal aperture can also occur independently from Ca^{2+} signalling, where SnRK2.6/OST1 becomes crucial in the activation of anion channels, such as SLAC1 (Geiger et al., 2009; Maierhofer et al., 2014), and K^+ efflux transporters like guard cell outward-rectifying K^+ (GORK) (Hosy et al., 2003). Additionally, SnRK2.6/OST1 inhibits K^+ importers, such as KAT1 (Sato et al., 2009), via phosphorylation and indirectly through transcriptional regulation. Moreover, OST1 activates ROS producers, RbohF and RbohD (Kwak et al., 2003; Sirichandra et al., 2009), key for ABA-induced ROS production and the activation of Ca^{2+} channels. Thus, OST1, whose knockout mutants show altered stomatal apertures (Acharya et al., 2013), plays a critical role in ABA-mediated stomatal movements. Under basal ABA levels, SnRK2s, Ca^{2+} importers and anion efflux channels are kept dephosphorylated, while a derepressed proton pump exports H^+ to the apoplast. As a result, the guard cell membrane is hyperpolarized and the K^+ rectifying channel is activated, facilitating the uptake of K^+ and maintaining guard cell turgor and the stomatal pore open. Guard cells are known to accumulate higher concentrations of basal ABA than any other leaf tissues, coupled with basal SnRK2 activity (Waadt et al., 2014; Zhang et al., 2020), supporting the notion that both ABA levels and signalling are key for ensuring proper stomatal conductance. Apart from local ABA activity, various other signals can regulate stomatal movements systemically by influencing ABA, whether through ROS or Ca^{+2} waves, or via small peptides such as CLE25 (reviewed in Mittler and Blumwald, 2015)(Takahashi et al., 2018).

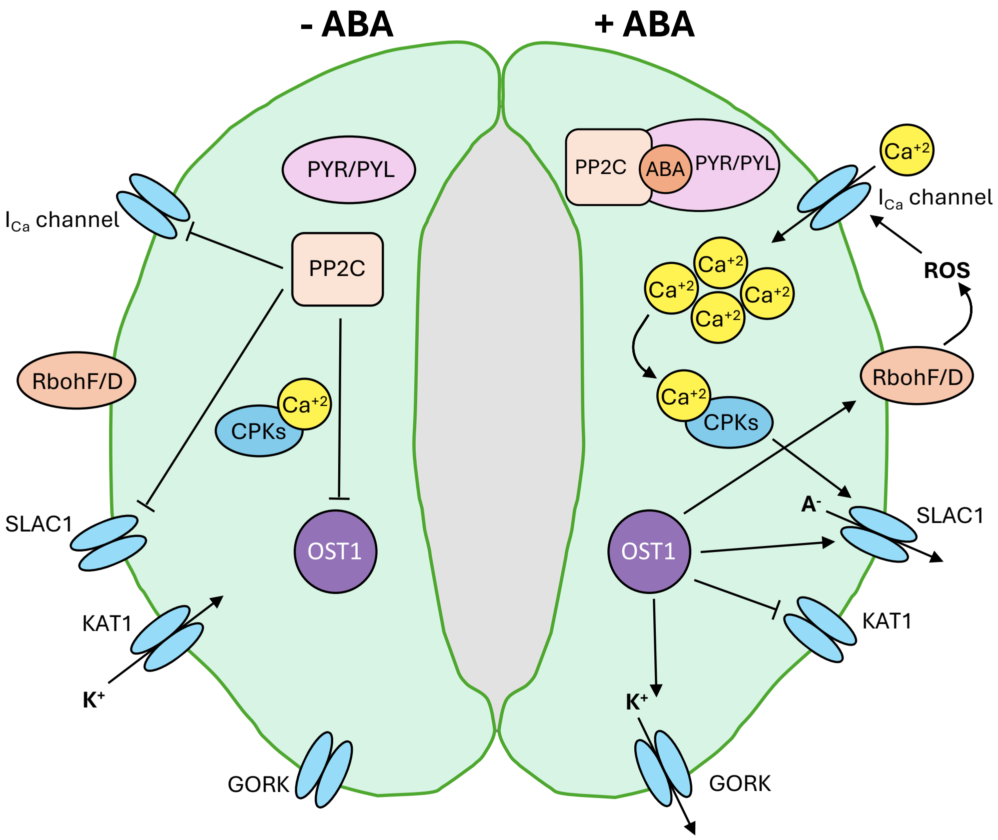

Figure 1.3. ABA signal transduction in guard cells.

Under basal ABA levels, SnRK2.6/OST1, Ca+2 importers, and anion (A-) efflux channels such as SLAC1 are kept dephosphorylated by PP2C phosphatases, while K+ rectifying channels like KAT1 are active. In the presence of ABA, ABA signalling is triggered, activating SLAC1, K+ exporters, and ROS producers RbohF/D, while repressing K+ importers. Ca+2 import channels are also derepressed from PP2Cs, activating CPKs that phosphorylate SLAC1. Figure adapted from Munemasa et al., 2015.

Stomatal aperture is an excellent proxy for the study of plant sensitivity to ABA treatment, as stomata play pivotal roles in functions tightly linked to the hormone, such as transpiration, photosynthesis, and the response to various stress. Considering that the size of stomatal pores is in the micrometre scale, direct measurements of their aperture require the use of microscopes. Guard cell movements can also be determined indirectly by monitoring leaf temperature, gas exchange rates, or fresh weight loss (Orzechowska et al., 2020).

1.2 Alternative splicing

Alternative splicing is a post-transcriptional mechanism that allows the production of multiple mRNAs from the same pre-mRNA through differential usage of splice sites. It is a crucial process in the regulation of gene expression and contributes to proteome diversity, enhancing fitness towards the varying environmental stress conditions (reviewed in Singh and Ahi, 2022). The most recent estimates indicate that ~60-80% of intron-containing genes in Arabidopsis undergo alternative splicing (Marquez et al., 2012a; Zhu et al., 2017a). Interestingly, research in plants over the last decades highlights a potential role for this mechanism in the regulation of ABA-mediated responses, both developmental and stress-related (reviewed in Staiger and Brown, 2013; Filichkin et al., 2015; Laloum et al., 2018; Punzo et al., 2020a; Yang et al., 2022; Sybilska and Daszkowska-Golec, 2023).

1.2.1 Mechanism and regulators

During alternative splicing, noncoding sequences (introns) are co-transcriptionally removed from the pre-mRNA, and different combinations of coding sequences (exons) are joined, resulting in different transcript isoforms. The splicing reaction, summarized in Figure 1.4, begins with the recognition of short consensus sequences, called splice sites, at the 5’ and 3’ ends of the intron and the branch point, near the 3’ splice site, by small nuclear ribonucleoproteins (snRNPs). Generally, splice sites close to the consensus sequence lead to constitutive splicing while divergent ones result in alternative splicing (reviewed in Kornblihtt et al., 2013). Next, the assembly of the spliceosome, an RNA-protein megacomplex, takes place on the pre-mRNA via sequential recruitment of snRNPs and auxiliary proteins. This is followed by a two-step transesterification reaction catalysed by the spliceosome to excise the intron and join the adjacent exons (reviewed in Kornblihtt et al., 2013).

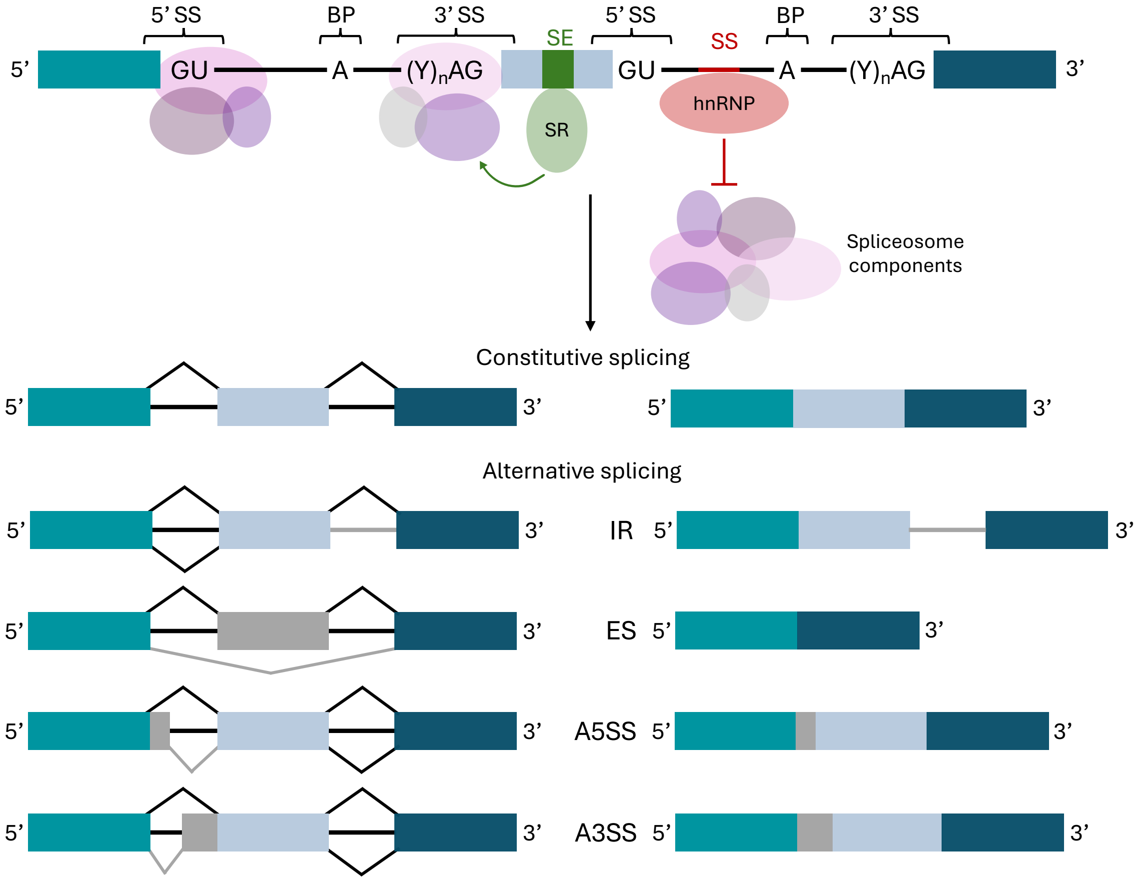

Figure 1.4. Alternative splicing: regulators and type of events.

Alternative splicing is regulated by *cis*-elements, including the 5’ and 3’ splice sites (5’SS, 3’SS), branch point (BP), splicing enhancers (SE), and splicing silencers (SS), as well as *trans*-acting regulators such as SR and hnRNP proteins. These factors influence spliceosome assembly and splice site recognition. The main types of alternative splicing events are intron retention (IR), exon skipping (ES), and alternative 5’ and 3’ splice sites. Figure adapted from (Laloum et al., 2018).

Depending on the sequence affected, alternative splicing events can be classified into four main types: intron retention (IR), exon skipping (ES), and alternative 5’ and 3’ splice sites (A5SS and A3SS) (see Figure 1.4). In plants, it is well established that IR is the most common type of alternative splicing event, while ES prevails in animals (reviewed in Chaudhary et al., 2019; Du et al., 2023). This is most likely due to divergent evolution of the process that led to differences in the gene architecture or in the regulatory elements controlling this mechanism (reviewed in Chaudhary et al., 2019; Du et al., 2023).

The selection of splice sites is controlled by both *cis*- and *trans*-acting elements. *Cis*-acting elements, such as exonic and intronic splicing enhancers and silencers, are sequences within the pre-mRNA that affect the recognition of nearby splice sites that promote or repress their recognition (reviewed in Reddy et al., 2013). On the other hand, *trans*-acting factors, such as serine/arginine-rich (SR), heterogeneous nuclear ribonucleoprotein (hnRNPs) or other splicing factors, are recruited to *cis*-regulatory sequences to control the usage of the adjacent splice sites (reviewed in Reddy et al., 2013). SR proteins, which in plants exist in higher numbers than in animals and include plant-specific members (reviewed in Kornblihtt et al., 2013), are central players in the regulation of alternative splicing, being involved in the early steps of spliceosome assembly, and are regulated not only by phosphorylation/dephosphorylation and signal transduction pathways, but also by splicing (reviewed in Jin, 2022).

Functionally, the outcome of alternative splicing depends on where in the pre-mRNA sequence the event takes place and can affect transcript stability, protein sequence and subcellular localization. If it occurs in the coding sequence (CDS), it may result in the production of different protein isoforms, potentially altering their function by affecting key domains or localization signals. It can also introduce premature termination codons (PTCs), which may produce truncated proteins with different functional activities, or regulate transcript abundance by triggering the degradation of the mRNA via the nonsense-mediated decay (NMD) pathway (Kalyna et al., 2012; Drechsel et al., 2013). On the other hand, if the event takes place in an untranslated region (UTR), it can either positively or negatively impact mRNA stability and translation efficiency (reviewed in Srivastava et al., 2018).

The regulation of alternative splicing depends not only on the strength of the splice sites (how close their sequence is to the consensus), the presence of enhancers and inhibitors, or the abundance of specific splicing factors (reviewed in Kornblihtt et al., 2013). Alternative splicing can also be regulated by transcription, as it has been shown that these two mechanisms occur simultaneously, and thus, processes like splice site choice can be modulated by the elongation rate of the RNA polymerase II (RNAPII), or via interactions between the splicing factors and the transcriptional machinery (reviewed in Kornblihtt et al., 2013; Reddy et al., 2013; Jabre et al., 2019). Therefore, any factors that affect transcription, such as changes in epigenetic marks (reviewed in Jabre et al., 2019), will also impact splicing. Despite this clear connection, transcriptomic and genome-wide association studies have suggested they act as two relatively distinct regulatory layers, with only a moderate overlap (~9-30%) of genes affected at the gene expression and splicing levels (Ding et al., 2014; Calixto et al., 2018; Chen et al., 2018b; Martín et al., 2021; Yu et al., 2021).

1.2.2 Evolution of alternative splicing

Alternative splicing was independently discovered more than 40 years ago by the laboratories of Sharp and Roberts, providing an explanation for the increased coding capacity of organisms from a given number of genes (reviewed in Berk, 2016). Initially regarded as an exception, the onset of new sequencing technologies revealed that alternative splicing is more prevalent than previously imagined. Nearly all intron-containing genes undergo alternative splicing in humans, up to 80% in plants, and with a wide range in fungi from 0.2% and 38.4% (Pan et al., 2008; Wang et al., 2008; Marquez et al., 2012a; Zhu et al., 2017a; Fang et al., 2020). Interestingly, studies in vertebrates found that the complexity of organisms was proportional to the level of alternative splicing (Barbosa-Morais et al., 2012; Chen et al., 2014), as it increases proteome functional diversity and facilitates rapid evolutionary transitions and ecological adaptation (reviewed in Singh and Ahi, 2022). Curiously, in Arabidopsis, alternative splicing plays a preponderant role in the response to stresses than in animals, where it mainly affects cell and tissue-related processes, probably due to differences in their lifestyle (Martín et al., 2021). Thus, the dynamic nature of alternative splicing in response to environmental stresses, along with its active regulation by various signalling pathways, allows plants to finely tune their physiological responses to environmental challenges and further demonstrates its evolutionary importance and its contribution to plant fitness (reviewed in Staiger and Brown, 2013).

1.2.3 Alternative splicing and the ABA pathway in the regulation of stress responses

Alternative splicing has emerged as an important mechanism for plant stress responses (reviewed in Staiger and Brown, 2013; Cui and Xiong, 2015; Filichkin et al., 2015; Laloum et al., 2018; Punzo et al., 2020a). It enables a rapid response to adverse environmental conditions without the need to go through transcription, a more time-consuming process (reviewed in Liu et al., 2022). Numerous studies have shown that exposure to abiotic stresses like drought, high salinity, or cold, triggers significant changes in alternative splicing (Ding et al., 2014; Feng et al., 2015; Thatcher et al., 2016; Calixto et al., 2018). Moreover, in plants stress regulators are prone to undergo alternative splicing (Ner-Gaon et al., 2004), suggesting the importance of this mechanism in the response to stress. In addition to being partially independent from transcription, alternative splicing has been suggested to represent a stress-specific layer of regulations, as multiple stresses show minimal overlap of alternative splicing events (reviewed in Punzo et al., 2020a).

ABA, a central hub for abiotic stress responses, in addition to inducing changes in gene expression (Song et al., 2016), has been shown to modulate alternative splicing and vice versa (reviewed in Yang et al., 2022). Multiple splicing factors are known to bind and regulate the splicing of ABA signalling components, and when plants lack such splicing factors, they display altered ABA responses. A well-studied case is the alternative splicing control of the PP2C HAB1 pre-mRNA by RBM25 (RNA-binding protein 25) and AtACINUS (Arabidopsis homolog of mammalian apoptotic chromatin condensation inducer in the nucleus), resulting in two protein isoforms with opposite roles in ABA-mediated functions, switching on and off the signalling pathway (Wang et al., 2015; Zhan et al., 2015; Bi et al., 2021). When *RBM25* or *AtACINUS* are absent, plants become hypersensitive to ABA during germination and seedling establishment (Wang et al., 2015; Zhan et al., 2015; Bi et al., 2021). *SAD1 (supersensitive to ABA and drought 1)* encodes a spliceosomal U6 snRNP component, LSm5, involved in splice-site recognition and their loss of function results in genome-wide splicing defects, including in *SnRK2.2*, and in ABA-hypersensitive phenotypes (Cui et al., 2014). The splicing factor SKIP (Ski-interacting protein) regulates the ABA pathway by controlling the alternative splicing of ABA signalling genes. Defects in the splicing of PYLs and ABFs, and of PP2Cs, stemming from loss of *SKIP* function, consequently repress or enhance, respectively, their expression resulting in ABA-hyposensitive plants (Zhang et al., 2022a). In addition to *SKIP*, *ABI5* splicing is also regulated by the splicing factor U2AF 65b, which upon mutation leads to an early flowering phenotype (Xiong et al., 2019). The alternative splicing of another ABA-insensitive transcription factor, *ABI3*, is controlled by DRT111 (DNA-damage repair/toleration protein 111) and SUA (suppressor of ABI3-5) which interact with the splicing factors SF1 and U2AF 65 to retain a cryptic intron of key importance at the germination stage (Sugliani et al., 2010; Punzo et al., 2020b). Mutants of both *sua* and *drt111* differ in their ABA sensitivity compared to the wild type (Sugliani et al., 2010; Punzo et al., 2020b).

The above examples of splicing factors regulating the splicing of key ABA genes, and whose loss-of-function mutants show altered ABA sensitivities, indicates the importance of this post-transcriptional mechanism in ABA signalling and ABA-mediated growth, development, and stress responses. Supporting this notion, chemical inhibition of splicing also triggers ABA-like responses via defects in the splicing of ABA pathway regulators such as PP2Cs (AlShareef et al., 2017; Ling et al., 2017).

Several studies have shown that plant SR proteins are transcriptionally regulated and change their splicing patterns in response to ABA (Palusa et al., 2007; Cruz et al., 2014). Indeed, loss-of-function mutants of SR proteins, such as RS40, SR45 or SCL30a, show altered ABA sensitivities (Chen et al., 2013; Carvalho et al., 2016; Laloum et al., 2023). However, it is important to note that metazoan SR proteins have been implicated in additional functions aside from pre-mRNA splicing, some being involved in mRNA export, transcript stability, or translation (reviewed in Jin, 2022), and thus the effect of the mutations could be due to mis-splicing of downstream targets or other non-canonical roles. Importantly, ABA treatment was shown to promote the usage of non-canonical splice sites (Zhu et al., 2017a). Another indication of the ABA-mediated regulation of alternative splicing comes from the SnRK2 ABA signalling core components, which target multiple splicing factors, such as BTR1L (binding to ToMV RNA 1, large protein product) or SCL30 (SC35-like splicing factor 30) (Umezawa et al., 2013; Wang et al., 2013). Thus, the interplay between ABA signalling and alternative splicing is key for the plant's ability to respond to ABA-mediated stresses.

1.3 Natural variation in stress responses

The model plant *Arabidopsis thaliana* is distributed throughout the Northern hemisphere, where the wide variety of climates suggests the occurrence of evolutionary processes to occupy new ecological niches. In addition to its short life cycle, its largely self-fertilizing nature, and its small genome, *A. thaliana* offers the advantage of having available numerous fully sequenced nearly homozygous lines, called accessions, from ecologically diverse habitats, making it an excellent tool to study genetic and phenotypic variation of plants responses to stress (Alonso-Blanco et al., 2016). Natural variation refers to the genetic differences present among individuals of the same species, which can manifest in multiple ways, including changes in one individual base, called single nucleotide polymorphisms (SNP), or insertions or deletions (indels) in the sequence. Numerous studies have made use of collections of Arabidopsis ecotypes, locally-adapted natural variants, to explore the variability in stress responses and uncover novel targets. Many of these studies focused on ABA-mediated stress responses, such as drought (Bouchabke et al., 2008; Zhang et al., 2008; Juenger et al., 2010; Imai et al., 2015a; Davila Olivas et al., 2017) and salt stress (Quesada et al., 2002; Baxter et al., 2010; Galpaz and Reymond, 2010; Katori et al., 2010; DeRose-Wilson and Gaut, 2011). *RAS1 (response to aba and salt 1)* and *AtHKT1 (high-affinity K^+ transporter 1)* are two examples of genes identified from natural variation studies on salt tolerance. *RAS1* was identified through quantitative trait locus (QTL) mapping conducted with recombinant inbred lines derived from salt-sensitive and salt-tolerant ecotypes, where the salt-tolerant allele presented multiple nucleotide variations, including a premature stop codon (Ren et al., 2010). The sodium transporter *AtHKT1* was initially detected in an ionomics study as the locus responsible for sodium accumulation (Rus et al., 2006), and later supported by a genome-wide association study (GWAS) study which detected its association with coastal and saline-soil inhabiting populations (Baxter et al., 2010). In 2017, Kalladan et al. found significant natural variation in ABA accumulation upon drought stress among nearly 300 Arabidopsis accessions. Other studies reported sequence polymorphisms in *NCED3*, a key gene in ABA biosynthesis, associated with increased ABA levels under drought conditions (Hao et al., 2009; Kalladan et al., 2019).

In plants, alternative splicing is known to be triggered by environmental cues, suggesting that ecotypes from diverse habitats could exhibit variations in splicing important for attaining phenotypic plasticity. Some studies have, in fact, identified differences in the splicing profiles of Arabidopsis natural variants (Gan et al., 2011; James et al., 2018; Khokhar et al., 2019a). For example, the sequencing and re-annotation of 18 Arabidopsis accessions genomes uncovered sequence variations affecting splice sites and contributing to the alternative splicing of thousands of genes (Gan et al., 2011). Khokhar et al., in a more recent study with over 600 accessions, found that sequence polymorphisms affecting splicing patterns were enriched in stress-responsive genes.

Natural variation can be studied from different approaches. the most used methods are QTL mapping and GWAS, powerful tools for the identification of genetic loci associated with phenotypic traits. However, they show limited resolution, are affected by population structure, and require a high number of lines to be performed. The combination of these methods with expression arrays or RNA sequencing, allows the study of genetic variants affecting expression (expression QTL [eQTL]) and splicing patterns (splicing QTL [sQTL]) (Khokhar et al., 2019a; Yu et al., 2021; Wu et al., 2022). Additionally, comparative transcriptomic analysis can be performed with natural variants with known contrasting phenotypes, where RNA-sequencing (RNA-seq) is used for in-depth studies of changes in the transcriptional or splicing response to stressors in order to identify important genes involved in stress responses (Li et al., 2020; Liu et al., 2020; Kong et al., 2021; Zhang et al., 2022b).

1.4 Thesis outline

Alternative splicing has emerged as a major contributor to plant stress tolerance, but the specific genes and events involved remain mostly unknown. Considering that different natural variants of the model plant *Arabidopsis thaliana* have differences in stress sensitivity and splicing profiles, we are making use of natural variance to substantiate the role for alternative splicing in stress responses mediated by ABA and to uncover functionally significant splicing targets.

In Chapter 1, I conducted a comprehensive review of the literature focusing on ABA, its signalling and metabolic pathways, as well as its functions in plants under normal conditions and in the context of stress. I also explore the role of alternative splicing in stress responses and its interaction with the ABA pathway. Finally, I describe natural variation in stress responses in Arabidopsis and explain how it can be used as a tool to uncover new targets.

This thesis aimed to accomplish three main objectives:

Objective 1: Identify natural variants with distinct ABA sensitivity

I conducted a preliminary phenotypic screen on a collection of 24 natural variants (Chapter 2). Parameters controlled by the hormone were tested, such as seed germination, root growth, and stomatal aperture, which led me to select the two final ecotypes for further analysis.

Objective 2: Develop a protocol for assessing stomatal aperture

While various techniques are available for the assessment of stomatal aperture, many entail the disruption of the plant tissue (such as blending or epidermal strips), its immediate imaging, or are limited in the samples' long-term preservation (Pei et al., 1997; Inoue et al., 2008; Monda et al., 2011; Zimmerli et al., 2012; Monda et al., 2016; Zhu et al., 2016). To circumvent these issues, we optimized a protocol (Chapter 3) based on surface leaf silicon impressions that offers numerous advantages: it is a quick, cost-effective, and durable method that allows multiplexing, preserves tissue integrity, and is adaptable to various species and parameters, such as stomatal density, size, or cellular patterns.

Objective 3: Comparative transcriptomic analysis

Once the final two ecotypes were selected, the main goal of the thesis was to determine molecular changes at the gene expression and splicing levels, and to identify new splicing targets that could explain the observed physiological differences in response to ABA. To achieve this, we performed an in-depth comparative transcriptomic analysis via RNA-seq on the two selected ecotypes with different ABA sensitivities (**Error! Reference source not found.**). We determined their endogenous ABA levels and conducted a thorough transcriptional characterization of their ABA metabolic and signalling pathways. I also assessed natural variance in selected targets and functionally characterized the genes whose splicing is regulated by ABA.

To conclude, Chapter 5 includes a final discussion reviewing the main findings of the thesis and their biological relevance. It also proposes future lines of work to address new questions arising from our results.

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Chapter 2. Preliminary Screening for Distinct ABA Sensitivity Among Arabidopsis Ecotypes

Author contributions:

All phenotypic assays and data analysis were performed by me. The selection of accessions, conceptual design of experiments, and data interpretation were supervised by Paula Duque. Plant care was managed by Vera Nunes.

2.1 Natural variation in the response to exogenous ABA

Arabidopsis thaliana, a major model plant species, is distributed across a wide range of natural habitats throughout the Northern hemisphere and counts with thousands of natural accessions that allow the study of intraspecific variation. Given that different Arabidopsis ecotypes show variations in stress sensitivity (Quesada et al., 2002; Bouchabke et al., 2008; Mckhann et al., 2008; Zhang et al., 2008; Baxter et al., 2010; Galpaz and Reymond, 2010; Juenger et al., 2010; Katori et al., 2010; DeRose-Wilson and Gaut, 2011; Gery et al., 2011a; Barah et al., 2013b; Barah et al., 2013a; Imai et al., 2015a; Takahashi et al., 2015; Davila Olivas et al., 2017; Fischer et al., 2017; Kalladan et al., 2017; Coolen et al., 2019; Schulz et al., 2021) and in their splicing profiles (Gan et al., 2011; James et al., 2018; Khokhar et al., 2019a), our project aimed at making use of natural variants to substantiate the role of alternative splicing in ABA-mediated stress responses.

Our first objective was to identify ecotypes with contrasting sensitivities to ABA and study ABA responses comprehensively by comparing the two extremes and an intermediate ecotype. We started with 24 *A. thaliana* ecotypes, representative of its geographic distribution, including two widely used accessions, Col-0 and Ler-0 (Table 2.1). All the chosen lines have genomic data available as part of the 1001 Arabidopsis Genomes Project, with the exception of Chi-1 and Kl-4 from Takahashi et al. (2015), which were included because of their ABA-insensitive phenotypes at the stomatal level.

Table 2.1. List of *Arabidopsis thaliana* natural accessions.

Name of accession, country, and coordinates of origin. Data collected from 1001 Genomes Project, The Arabidopsis Information Resource (TAIR), and Versailles Arabidopsis Stock Centre databases.

Name	CS Number	Country	Latitude	Longitude
Altai-5	CS76433	CHN	47.75	88.4
Anz-0	CS76439	IRN	37.47	49.47
Ara-1/Sha	CS76382	AFG	37.29	71.3
Bur-0	CS76734	IRL	54.1	-6.2
C24	CS76106	POR	41.2	-8.4
Can-0	CS76740	ESP	29.2144	-13.4811
Chi-1	CS28137	RUS	53.75	34.73

Col-0	CS70000	POL	52.74	15.24
Ct-1	CS76786	ITA	37.3	15
Cvi-0	CS76789	CPV	15.1111	-23.6167
Edi-0	CS76831	UK	55.9494	-3.16028
Ga-0	CS76490	GER	50.3	8
Ge-0	CS76875	SUI	46.5	6.08
Kl-4	CS28393	GER	50.95	6.966
Kn-0	CS76969	LTU	54.8969	23.8924
Ler-0	CS77020	GER	47.984	10.8719
Map-42	CS77732	USA	42.166	-86.412
Mh-0	CS76550	POL	50.95	20.5
Oy-0	CS77156	NOR	60.385543	6.193019
Sf-2	CS77247	ESP	41.7833	3.03333
St-0	CS77270	SWE	59	18
Strand-1	CS77284	NOR	68.8	15.45
Tsu-0	CS77389	JPN	34.43	136.31
Ws-0	CS1602	RUS	52.3	30

All seed stocks except for Col-0 and Ler-0, already available in our lab, were ordered from NASC (Nottingham Arabidopsis Stock Centre). Prior to the start of the project and the phenotypic screening, all lines were bulked and grown for two generations to eliminate any residual “stress memory” that could have been acquired in previous generations and to acclimate them to our laboratory conditions (Wibowo et al., 2016). All plants were grown at 20°C, 60% relative humidity, under a long-day photoperiod (16 hours light, 8 hours dark), unless specified otherwise.

A preliminary assay for seed germination, a process tightly regulated by ABA (Finkelstein, 2013), was conducted on 17 ecotypes to assess variation in sensitivity to ABA and identify those that differ significantly in their response to the hormone. Of the remaining seven ecotypes, six were discarded for exhibiting extremely delayed flowering due to lack of vernalization and difficulties in synchronizing seed availability for the assay. Can-0 was also later removed due to an issue with the original stock.

The results from the seed germination assay showed a wide range of responses among the ecotypes, with an intermediate response for the reference accession Col-0 in the higher ABA concentration (see Figure 2.1). Hereafter, the sensitivity of an ecotype to ABA is defined based on its relative response to Col-0.

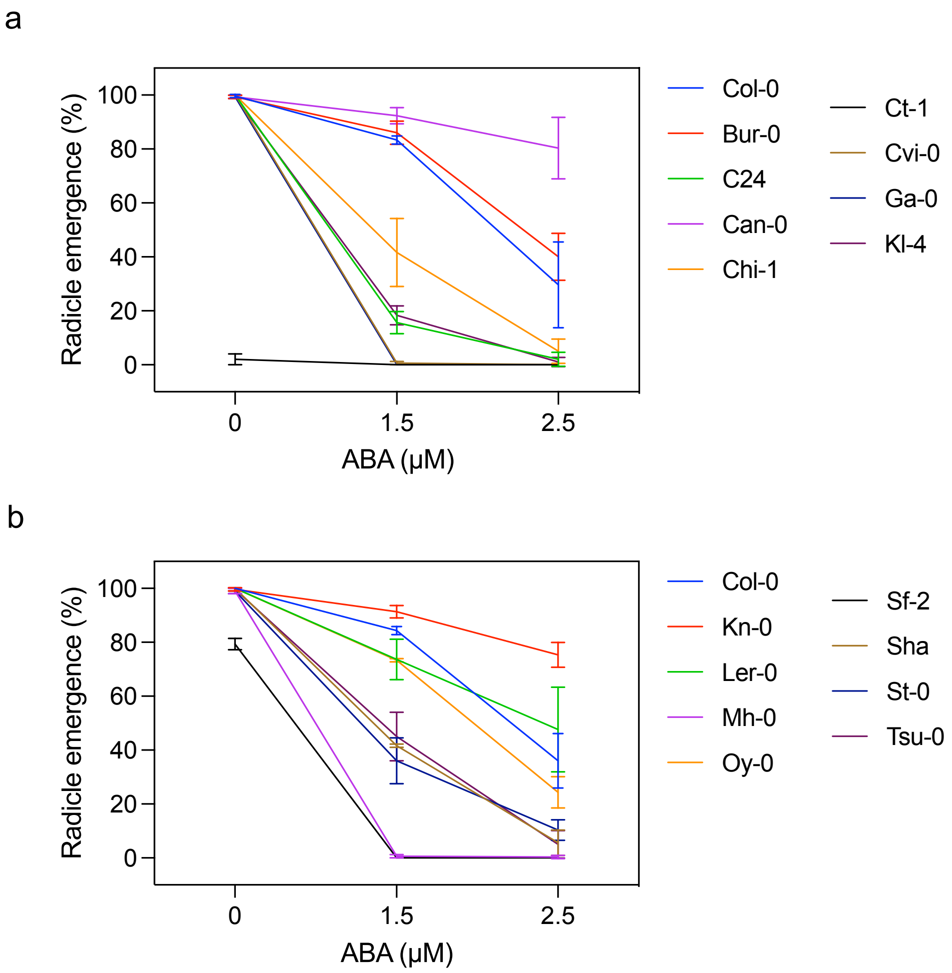

Figure 2.1. Germination assay.

One week-old seeds were surface-sterilized and stratified at 4ºC for 3 days in tubes with water. Seeds were then plated on either control or ABA-treated medium and germination rates (means ± SD, *n* = 3) were scored after 7 days. The germination of the 17 ecotypes was assessed in parallel over two sets (a and b), with Col-0 included in both sets as a reference.

Kn-0 ecotype was selected for further assays due to its hyposensitivity to ABA, and Mh-0, Ga-0, Cvi-0 and C24 for their hypersensitive response to ABA, all relative to Col-0 (see Figure 2.1). Sf-2 and Ct-1 were discarded due to their marked delay in seed germination under control conditions (see Figure 2.1).

A second round of screening for ABA sensitivity was conducted by examining lateral root density, a process inhibited by ABA. This allowed me to narrow down the ecotype cohort to Kn-0, Cvi-0, C24 and Mh-0, which showed the most divergent responses from Col-0, although at different ABA concentrations (see Figure 2.2).

After conducting a final screen of the stomatal aperture response to ABA for the selected ecotypes in two experiments with varying conditions (see Figure 2.2), I discarded Cvi-0 and Mh-0 for not differing from Col-0. C24, which was present in both experiments, was also discarded as it exhibited inconsistent behaviour.

We therefore decided to proceed with further experiments, performing the proper biological replicates, using the Kn-0 and Col-0 natural variants. This decision was based on the consistent hyposensitive phenotype of Kn-0 and the absence of a consistent hypersensitive ecotype across the different assays. Although the initial approach was to work with the reference accession, Col-0, and two other ecotypes (one ABA-hypersensitive and one ABA-hyposensitive compared with Col-0), the lack of consistency across the assays resulted in the selection of only one additional ecotype besides Col-0.

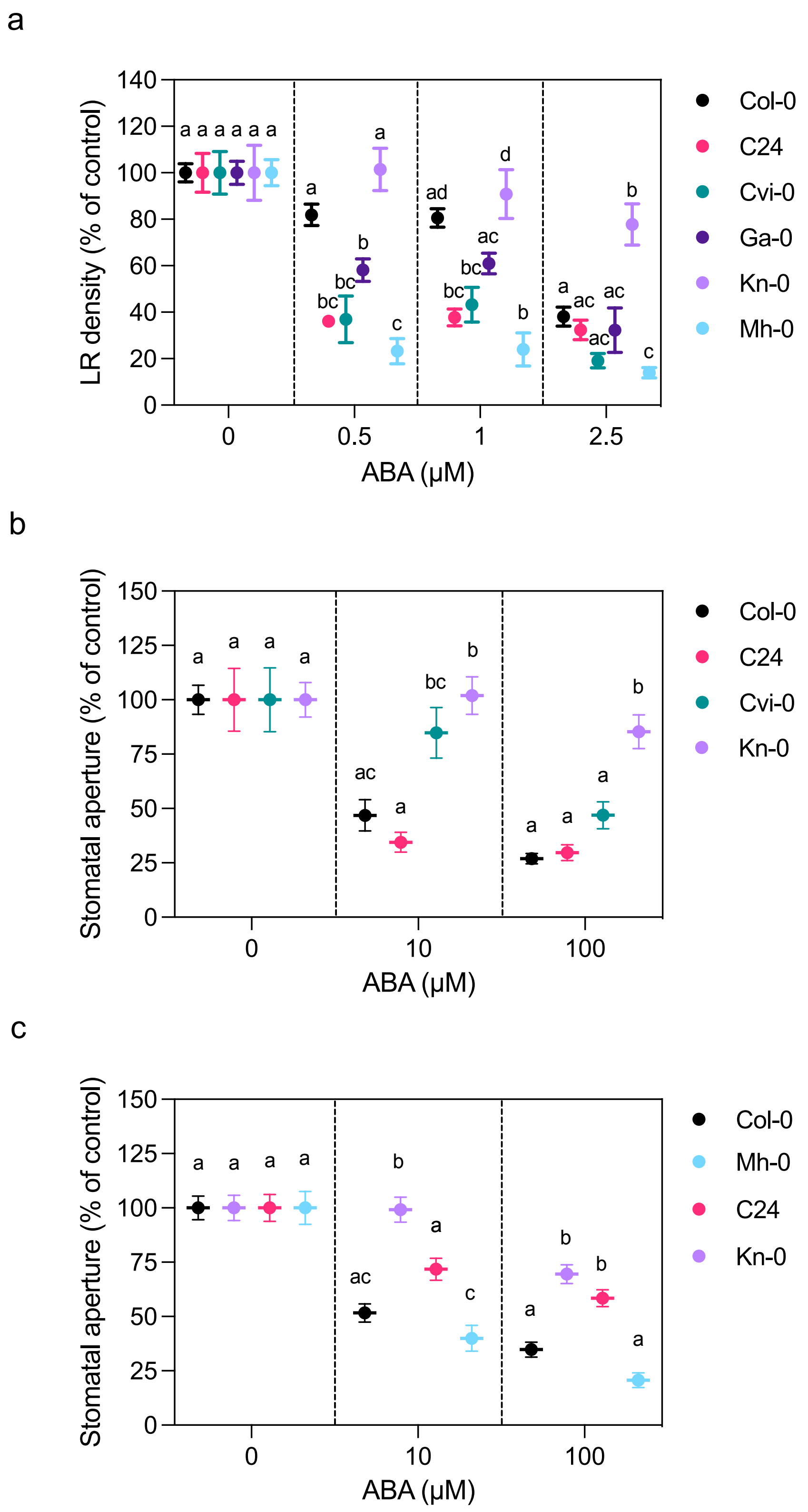

Figure 2.2. Lateral root and stomatal aperture assays.
(a) Lateral root density (number of lateral roots per cm of primary root) scored 11 days post transfer of 6-day-old seedlings to either control or ABA-treated plates.

(b, c) Stomatal pore aperture (pore width/pore length) scored at (b)18 and (c) 9 days old after 2 hours in control or ABA-treated medium. Data presented as mean ± SD with *n* = 6-16 for (a), and as scatter dot plots for (b) and (c) with n = 13-88. Letters denote statistically significant differences, with $p < 0.05$ (two-way ANOVA with Tukey HSD test).

The preliminary results shown above were conducted without the required biological replicates and as such should be interpreted with caution. Nonetheless, we identified Kn-0 as an ecotype that potentially differs significantly in its response to ABA compared with Col-0, which, to our knowledge, has not been previously reported. Earlier studies on stress responses using Kn-0 and Col-0 have shown differences in their tolerance to salt, drought, and freezing conditions (Quesada et al., 2002; Bouchabke et al., 2008; Mckhann et al., 2008; Zhang et al., 2008; Katori et al., 2010; Gery et al., 2011a). **Error! Reference source not found.** investigates the differences in ABA sensitivity identified here between these two Arabidopsis accessions at greater depth.

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Chapter 3. Assessing Abscisic Acid-Mediated Changes in Stomatal Aperture Through High-Quality Leaf Impressions

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Author contributions:

Protocol optimization experiments were conducted by me. All images, including microscopy images, and all figures were captured and designed by me, respectively. The writing and development of this protocol were carried out under the joint supervision of Rosanna Henriques and Paula Duque.

3.1 Abstract

Plants live in highly dynamic surroundings and need to cope with constant environmental challenges. In order to do so, they developed quick reactions to stress which allow them to gain time while mounting a major response. In this first line of defence are the stomata, leaf epidermal pores in charge of regulating water loss and photosynthesis. Stomatal movements are controlled by the stress phytohormone abscisic acid (ABA), which induces fast closure of the stomata upon perception of stress conditions. By modulating plasma membrane ion channels, ABA leads to loss of water by the guard cells surrounding the stomatal pore and a consequent reduction of its aperture. Here we provide a microscopy-based method to assess the plant's response to ABA through measurements of the stomatal aperture. This protocol describes a simple, quick and unexpensive method to prepare high-quality impressions of leaves of *Arabidopsis thaliana* seedlings from long-lasting silicone-based casts, allowing detailed imaging and accurate determination of the aperture of stomatal pores.

3.2 Introduction

Plant growth is severely affected by adverse external conditions, biotic and abiotic, imposing severe detrimental effects on agriculture. The phytohormone abscisic acid (ABA), involved in multiple developmental processes, is widely recognized as a major regulator of plant responses to stress. When plants perceive environmental challenges, such as drought, high salinity or pathogen attack, cellular ABA levels increase sharply, triggering downstream signalling and implementing a plethora of adaptive responses. These can be either long-term morphological adaptations, or rapid physiological responses such as the control of stomatal movements, which occurs within just a few minutes (reviewed in Finkelstein et al., 2002; Finkelstein, 2013; Munemasa et al., 2015). Stomata are microscopic pores located in the epidermis of the aerial parts of plants through which gases are exchanged, which play a crucial role in balancing photosynthesis and water loss through modulation of their aperture.

Stomatal regulation is controlled by core ABA signalling components that activate or repress the activity of ion channels at the plasma membrane of guard cells, altering their turgor pressure. This also involves reorganization of the cytoskeleton and vesicle trafficking to cope with the changes in guard cell volume, as well as alterations in gene expression specific to these processes (reviewed in Finkelstein, 2013). Upon binding to its cytosolic receptors, ABA forms a complex with PP2C (Type 2C Protein Phosphatases) phosphatases, thus releasing the inhibition of downstream SnRK2 (SNF1-related protein kinase2) kinases (Munemasa et al., 2015). One of the consequences of phosphorylation of SnRK2 targets is the activation of Ca^{2+} channels on the plasma membrane, resulting in an increase in intracellular Ca^{2+} and activation of Ca^{2+}-dependent kinases, which in turn activate anion channels inducing a large efflux of anions (Hewage et al., 2020). This causes depolarization of the plasma membrane and activation of K^+ outward rectifying channels, with ABA also inhibiting the plasma membrane proton pump and K^+ inward rectifying channels. The net result is a massive efflux of anions and cations accompanied by loss of water from guard cells, which reduces their turgor pressure and thereby the aperture of the stomatal pore (reviewed in Finkelstein et al., 2002; Munemasa et al., 2015; Hewage et al., 2020).

Stomatal closure can also occur systemically via the promotion of ABA biosynthesis across the plant by means of ROS (Reactive Oxygen Species)/Ca^{2+} waves that propagate from local to systemic leaves or through small peptides like CLE25 (for CLAVATA3/EMBRYO-SURROUNDING REGION-related protein 25), which was recently found to efficiently disseminate water-deficiency signals from roots to leaves through the vasculature (reviewed in Takahashi and Shinozaki, 2019).

Rapid responses to stress cues, such as the closing of stomata, are crucial to plant survival as they help minimize harmful effects early in the exposure stage while the plant prepares larger magnitude responses to acclimate to the adverse conditions (reviewed in Kollist et al., 2019). ABA regulation of stomatal aperture is a well-established system for plant stress response studies. There are two main approaches to determine changes in stomatal aperture — indirect measurement of leaf transpiration through evaluation of leaf temperature or gas exchange rates, for which specialized equipment is required, or direct examination of the pore aperture by light microscopy (Eisele et al., 2016). Here we describe a microscopy-based method to assess ABA-induced changes in the stomatal aperture of Arabidopsis seedlings by creating high quality impressions of the surface of the primordial leaves, called cotyledons, through casts made with dental resin. Other direct methods available to score stomatal movements imply either the disruption of tissues or making impressions that require immediate imaging. Our protocol allows obtaining optimal resolution images from impressions of long-lasting *Arabidopsis thaliana* leaf surface casts, thus capturing a precise moment in stomatal aperture. Importantly, these imprints can be generated at different stages of leaf development in distinct species and can also be used to characterize other parameters such as leaf epidermal cell sizes or stomatal density and patterning. This is therefore a simple, quick, inexpensive and versatile method with broad applications in plant research.

3.3 Materials

3.3.1 Plant Material

1. *Arabidopsis thaliana* 7-10 day-old seedlings of the genotypes of interest (*see* **Note 1**).

3.3.2 Reagents and Solutions

1. Murashige and Skoog (MS) medium (Murashige and Skoog, 1962): 1x MS basal salt mix supplemented with 0.01% (w/v) myo-inositol and 0.05% (w/v) 2-(N-morpholino) ethanesulfonic acid (MES), pH adjusted to 5.7 with KOH 1 M in sterile distilled water. Add 0.8% (w/v) ultrapure agar, autoclave and pour plates.
2. Bleach-based sterilization solution: 10% v/v sodium hypochlorite and 0.25% (v/v) 20 % SDS in sterile distilled water. Prepare freshly.
3. Stomatal opening solution: 50 mM KCl, 10 mM MES pH 6.15 adjusted with 1 M KOH (*see* **Note 2**).
4. (S)-Abscisic acid (Duchefa Biochemie): 50 mM stock solution in absolute ethanol (*see* **Note 3**).

3.3.3 Consumables

1. Square Petri dishes (12 cm x 12 cm)
2. Micropore tape
3. 2-mL Eppendorf tubes
4. 6-well plates (*see* **Note 4**)
5. Absorbent paper
6. Parafilm
7. Microscope slides
8. Coverslips

3.3.4 Imprinting material

1. Silicone-based dental impression material Aquasil Ultra LV Regular 50 mL cartridges (Dentsply) (*see* **Note 5**)
2. Dispensing gun for 50-mL cartridges (Dentsply) (*see* **Note 5**)
3. Mixing tips for cartridges (Dentsply) (*see* **Note 5**)
4. Clear nail polish

3.3.5 Equipment

1. Climate-controlled growth cabinet set to long-day conditions (16 h light, 22°C / 8 h dark, 18°C) and 60% relative humidity, with cool-white light (100 µmol m^{-2} s^{-1}). Other conditions can also be used, accordingly to the required experimental set-up.
2. 4°C refrigerator
3. Laminar flow hood
4. Platform shaker
5. Fine forceps
6. Bright field microscope equipped with a camera for image acquisition (*see* **Note 6**).

7. ImageJ2 (Rueden et al., 2017) or equivalent image-processing software.
8. GraphPad Prism v 8.4.3 (San Diego, California) or equivalent statistical and data analysis software.

3.4 Methods

Workflow of the full process is represented in Figure 3.1.

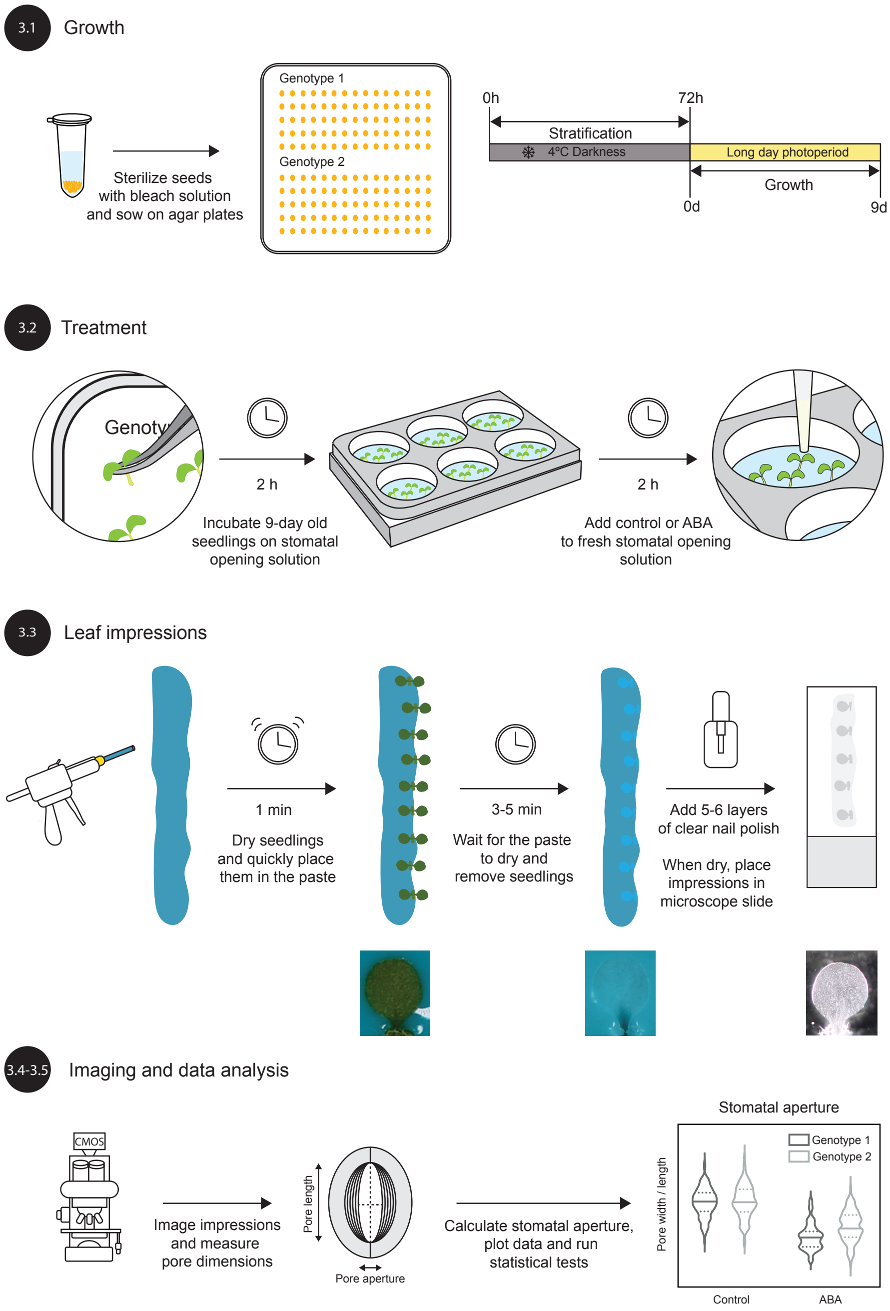

Figure 3.1. Overview of the experimental protocol.

Numbers correspond to the methods subheadings.

3.4.1 Plant Growth

1. Surface-sterilize seeds in 2ml Eppendorf tubes with bleach solution for 10 minutes under continuous, mild shaking and rinse 5-6 times with sterile distilled water (*see* **Note 7**).
2. Under sterile conditions, plate seeds on MS medium Petri dishes and stratify for 3 days at 4°C in the dark (*see* **Note 8**).
3. Incubate plates in a controlled-growth cabinet set to long-day conditions for 7-10 days (other conditions can be used, accordingly to the experimental set-up of interest).

3.4.2 ABA Treatment

4. After 7-10 days of incubation, carefully transfer 10-15 seedlings per genotype per treatment to 6-well plates (1 well per treatment per genotype) filled with 3-5 ml of stomatal opening solution (and incubate for 2 hours in growth cabinet under long-day conditions (*see* **Notes 9 and 10**).
5. After 2 hours, change the solution from the wells carefully (avoiding damage to the cotyledons) with fresh stomatal opening solution supplemented or not with ABA at the desired concentration, and incubate the plant material for another 2 hours in the light (*see* **Notes 11 and 12**).

3.4.3 Cotyledon Impressions

Figure 3.2 shows the materials and equipment used to make the cotyledon impressions.

Figure 3.2. Materials used for the preparation of leaf casts and impressions.

(1) Dispensing gun, (2) 50-mL cartridge, (3) mixing tips, (4) forceps, (5) clear nail polish, (6) microscope slide.

6. Prepare a fixed area at the bench to perform the impressions of the plant material and divide the space according to the number of different samples (*see* **Note 13**).

7. Load the dispensing gun with the cartridge containing the impression material and insert the mixing tip (*see* **Note 14**).
8. From each sample, carefully remove from the wells 10-15 seedlings and place them on top of absorbent paper to quickly remove excess solution and potential drops that could remain on the cotyledons (*see* **Note 15**).
9. Quickly apply one streak of the silicone paste on a fixed surface and carefully place each seedling on top, ensuring that the cotyledons are firmly in contact with the paste. Once all the seedlings from a sample are in place, and while the paste dries, start with the set of seedlings from the next sample (*see* **Note 16-18)**.
10. After 5 minutes, or after the paste becomes hard, remove the seedlings (*see* **Note 19)**.
11. Once the casts are ready, apply a thin layer of clear nail polish, avoiding bubbles, especially in this first layer, and allow to dry. Then repeat for 3-4 layers, making sure the nail polish has dried well before applying a new layer.
12. After the nail polish impressions are done, carefully remove them from the silicone paste with forceps and place them on a microscope slide, with the side that was in contact with the plant material facing up. Add a coverslip on top, gently pressing on the impressions, and secure it firmly by adding tape on the sides (*see* **Notes 20-21)**.

3.4.4 Image acquisition

13. Image the samples on a bright-field microscope (*see* **Notes 22-25)**.

3.4.5 Data analysis

14. Open the captured images on ImageJ and, if needed, change the length unit from pixels to µm taking into account the specific conversion factor from the objective used.
15. For each stoma, measure the pore width and pore length as shown in Figure 3.1. To calculate the stomatal aperture, the pore width is divided by the pore length.
16. Plot the data on GraphPad and apply the appropriate statistical test to determine significant differences between samples (*see* **Notes 26 and 27)**.

At least three independent experiments should be conducted for statistical accuracy. Representative images of impressions from wild-type Arabidopsis plants from the Columbia-0 (Col-0) ecotype treated with control and ABA solutions are shown in Figure 3.3, as well as an overview image of the leaf surface used for scoring epidermal cell sizes or stomatal density and patterning.

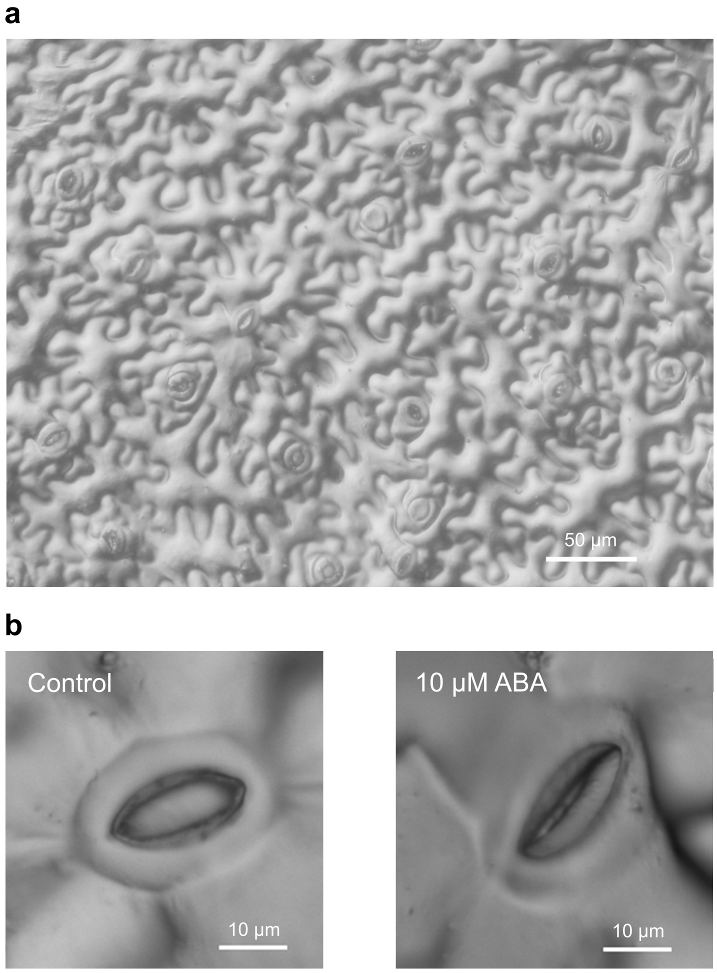

Figure 3.3. Examples of wild type (Col-0) Arabidopsis impressions.

(a) Overview of leaf epidermal cells from 21-day old Col-0 plants grown under LD conditions. Scale bar refers to 50 µm.

(b) Detail of stomata from leaf epidermis of seedlings grown under LD conditions and treated with control and ABA solutions as described in our protocol. Scale bars refer to 10 µm.

3.5 Notes

1. Leaves from plants at other developmental stages and from different species can be used.
2. Variations of the stomatal opening solution exist, some containing different ionic compounds or with altered ratios, but all induce hyperpolarization of the plasma membrane of guard cells to fully open the stomata.
3. ABA dissolves easily and should be stored at -20 °C. It is convenient to make aliquots to prevent recurrent thawing and freezing that may damage the stability of the hormone.

4. Any container with sufficient depth can be used for incubation of the plant material in the buffer. For small seedlings, plates with wells or even tubes could be used, while for larger plants Petri dishes are recommended.
5. To obtain the impressions, we use a two-component silicone-based material that comes in cartridges that maintain the two components in separate tubes. Thus, to apply this paste special tips are needed that will fully mix both components along its spiraled-shaped body, as well as a dispenser gun onto which to mount the cartridge (see Figure 3.2).
6. The images in Figure 3.3 were acquired on a Zeiss Imager Z2 + ApoTome.2 equipped with a Hamamatsu Orca Flash 4.0 v2 CMOS camera, using a 20x objective plus a 1.6x Optovar.
7. For discarding the solution between the washes, it helps to use a minicentrifuge to spin down the seeds.
8. Plate 20-30 seeds per genotype per treatment to ensure enough leaf material and, preferentially, plate all genotypes in the same plate to reduce variation between samples as much as possible.
9. Experiments should be conducted with plants of similar size and, although more relevant for older plants, the same pair of leaves should be used across samples. To transfer young seedlings, tweezers with curved tips are better to prevent cotyledon damage. Enough solution should be added to the container to allow for the plants to float. Large leaves can be cut off from the plant and Petri dishes used to make them float on the buffer.
10. While the first incubation takes place, prepare fresh stomatal opening solutions for the next step adding or not the appropriate ABA concentration (control or ABA treatment).
11. Stomatal movements are under the regulation of the circadian clock, making it crucial to consistently apply the ABA at the same time of day. There is evidence that the best time to perform this experiment is in the early afternoon (ZT7 for 12 h light/ 12 h dark days), when stomata are more responsive to ABA (Robertson et al., 2009).
12. The assay may need some rounds of optimization, and pilot experiments are good opportunities to test different ABA concentrations.
13. We use parafilm secured with tape as a fixed surface to apply the silicone streaks on top. It is convenient to previously delimit this surface and label the spaces onto which the different strips will be applied for each sample. However, we also recommend reusing plasticware materials, such as washed Petri dishes lids. The silicone can be applied directly on the lids, which can also be easily labelled and subsequently stored in boxes.

14. The dental product consists of two different pastes that are mixed inside the tip, so as soon as it leaves the cartridge it will start drying. After 2:15-2:45 minutes it will not be possible to apply more paste with the tip and a new one will need to be used. Dental resins from different brands will have different timings, so it is very important to follow each manufacturer's instructions.
15. Use small portions of tissue paper to easily remove any drops on the surface to cast. This step is very important to obtain detailed impressions of the leaf surface.
16. Because Arabidopsis' cotyledons lack trichomes, impressions of any of the sides can be done, preferentially of the side that is flatter. However, unlike cotyledons, true leaves in Arabidopsis have trichomes on the adaxial side, which can complicate visualization of the stomata, so the abaxial surface is recommended. When using leaves of larger area, the paste can be applied directly on top of the abaxial leaf surface.
17. When encountering problems to place the seedling such as both cotyledons are on the paste, and provided there is enough plant material, the seedlings can be held with the forceps by one of the cotyledons so that the other, with a flatter surface, stays firmly in contact with the silicone paste.
18. Large numbers of samples make the organization of the experiment crucial. The order in which the samples are transferred to the stomatal opening solution needs to be kept the same as that of the treatments and impressions, so all samples incubate the same amount of time in each of the solutions. It is also important to account for the amount of time that it will take to process each sample to organize the experiment in a gradual manner.
19. If the seedlings are left for too long in the resin, the plant tissue will dry and become harder to remove. There is then the possibility that some green tissue remains attached, which will render nail polish application problematic.
20. Although the coverslip is not necessarily required to image the impressions on a dry objective, it is useful to help them stay in place. If no coverslip is being used and the impressions are stiff and not sticking to the microscope slide, the borders can be gently pressed against the glass with a finger while using gloves, or nail polish applied on the borders to keep the impressions attached to the slide. When using an immersion objective, a coverslip must be added.
21. The silicone casts can be stored for years in case the nail polish impressions need to be repeated. The latter can also be kept intact for a long time.
22. Depending on the samples and species used, different magnifications may be needed. For Arabidopsis seedlings, at least a 20x objective should be used for scoring stomatal density or epidermal cell areas, and a minimum of 40x magnification when measuring stomatal aperture.

23. Because the impressions are transparent, contrast must be increased by manipulating light intensity, condenser aperture, and exposure time. When available, differential interference contrast (DIC) or oblique illumination could also be used.
24. As the leaf surface is not completely flat, the same area must be imaged at different focal distances, for which the z-stacks could the useful.
25. For each genotype and treatment, a total of 100-200 stomata should be photographed, preferentially from different leaves.
26. Violin plots are a good choice to visualize the entire distribution of the data while also displaying standard key statistical parameters like the median, range and quartiles.
27. If there are no significant differences in the stomatal pore length between samples, the pore width alone can be plotted.

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Chapter 4. Alternative Splicing as a Driver of Natural Variation in Abscisic Acid Response

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Author contributions:
The concept of the study and research designed were developed by Paula Duque and me. All experiments were performed by me, except for the protein quantification experiment, which was carried out by Dora Szakonyi, and the ABA measurements and dormancy assays, which were conducted by Jorge Lozano-Juste. RNA-seq data analysis was performed by me, and all figures were created by me and supervised by all authors. All authors contributed to the interpretation of the results.

4.1 Abstract

Abscisic acid (ABA) is a crucial player in plant responses to the environment. It accumulates under stress, activating downstream signalling to implement molecular responses that restore homeostasis. Natural variance in ABA sensitivity remains barely understood, and the ABA pathway has been mainly studied at the transcriptional level, despite evidence that post-transcriptional regulation, namely via alternative splicing, contributes to plant stress tolerance. Here we identified the Arabidopsis accession Kn-0 as less sensitive to ABA than the reference Col-0, as shown by reduced effects of the hormone on seedling establishment, root branching and stomatal closure, as well as by decreased induction of ABA marker genes. An in-depth comparative transcriptome analysis of the ABA response in the two variants revealed lower expression changes and fewer genes affected for the least ABA-sensitive ecotype. Notably, Kn-0 exhibited reduced levels of the ABA-signalling SnRK2 protein kinases and lower basal expression of ABA-reactivation genes, consistent with our finding that Kn-0 contains less endogenous ABA than Col-0. ABA also markedly affected alternative splicing, primarily intron retention, with Kn-0 being less responsive regarding both the number and magnitude of alternative splicing events, particularly exon skipping. We find that alternative splicing introduces a more ecotype-specific layer of ABA regulation and identify ABA-responsive splicing changes in key ABA pathway regulators that provide a functional and mechanistic link to the differential sensitivity of the two ecotypes. Our results offer new insight into the natural variation of ABA responses and corroborate a key role for alternative splicing in implementing ABA-mediated stress responses.

4.2 Introduction

Plants are constantly faced with challenging environmental changes to which they must adapt by responding to the severity of the stress accordingly. Besides playing a key role in developmental processes, such as seed dormancy and germination or the transition to flowering, the phytohormone abscisic acid (ABA) is required for an adequate response to abiotic factors that impose osmotic stress. In fact, when plants are faced with drought, high salinity or extreme temperatures, endogenous ABA levels increase sharply, triggering a wide range of physiological responses to increase stress tolerance (Finkelstein, 2013; Chen et al., 2020).

Inside the cell, ABA binds to a family of cytosolic receptors, the PYR (pyrabactin resistance)/PYL (PYR-like)/RCAR (regulatory component of ABA receptor) proteins, which interact with type 2C protein phosphatases (PP2Cs) to relieve their inhibition of SNF1-related protein kinases 2 (SnRK2s) (Ma et al., 2018; Maszkowska et al., 2021). SnRK2s are then able to phosphorylate downstream targets, thereby activating ABA signalling that can subsequently be enhanced or attenuated via different feedback mechanisms (Ali et al., 2020; Coego et al., 2021; Maszkowska et al., 2021). Fine-tuning of the pathway can also be achieved either by altering ABA transport or balancing its production with catabolism to modulate the levels of the active hormone through two main catabolic routes: irreversible hydroxylation or reversible conjugation of ABA (Ma et al., 2018). Indeed, in addition to the multistep pathway of *de novo* biosynthesis, ABA can be generated in the cell by means of reactivation of glucose-conjugated ABA, *i.e.* ABA-glucosyl ester (ABA-GE), allowing a more rapid response to prevent severe damage to the plant (Ma et al., 2018; Kollist et al., 2019).

Owing to the massive changes in gene expression and the crucial phosphorylation events involved, research on the ABA pathway has focused primarily on transcriptional and post-translational processes (Fujita et al., 2011; Song et al., 2016; Yang et al., 2017; Lim et al., 2022). Yet, our understanding of plant stress responses is quickly expanding to encompass regulation at the RNA level. A post-transcriptional mechanism emerging as a crucial player in plant stress tolerance is alternative splicing, which allows the production of multiple transcripts per gene (Laloum et al., 2018; Punzo et al., 2020a). Apart from a potent generator of proteomic diversity, this process is also a versatile means of modulating gene expression that can represent an important layer of stress response regulation independent from transcription (Ding et al., 2014; Calixto et al., 2018; Chen et al., 2018b; Martín et al., 2021). In *Arabidopsis thaliana* (Arabidopsis), alternative splicing occurs in 60-83% of intron-containing genes, varying like in animals across tissues and development but being used disproportionally in response to stress (Martín et al., 2021). More recently, alternative splicing is also being implicated specifically in the control of ABA responses, with many mutants in splicing factor genes displaying altered ABA sensitivities and hence defective stress responses (Laloum et al., 2018; Punzo et al., 2020b; Bi et al., 2021; Hong et al., 2021; Zhang et al., 2022a; Laloum et al., 2023). Moreover, treating plants with splicing inhibitors has been reported to induce ABA-like responses (AlShareef et al., 2017; Ling et al., 2017), and ABA exposure promotes the usage of noncanonical splice sites (Feng et al., 2015; Zhu et al., 2017b), corroborating a role for the hormone in mediating the splicing response to stress.

Studies of the ABA pathway and ABA-mediated stress responses have relied mainly on the analysis of mutant lines (Finkelstein, 2013). However, an increasing number of reports in the last decades makes use of collections of locally-adapted natural variants, *i.e.* ecotypes, to unravel the genetic variations underlying the diversity in the response to stress, namely in the many Arabidopsis accessions available, an invaluable resource that has allowed the identification of novel players in plant stress tolerance (Assmann, 2013; Yolcu et al., 2020). Both quantitative trait locus (QTL) analyses and genome-wide association studies (GWAS) have uncovered great variability in the response to ABA-mediated stresses, including drought (McKhann et al., 2004; Bouchabke et al., 2008; Des Marais et al., 2012; Imai et al., 2015b; Kalladan et al., 2017; Kalladan et al., 2019), high salinity (Quesada et al., 2002; Baxter et al., 2010; Galpaz and Reymond, 2010; Katori et al., 2010; DeRose-Wilson and Gaut, 2011; Julkowska et al., 2016) and freezing temperatures (Mckhann et al., 2008; Gery et al., 2011b). An early microarray analysis (Chen et al., 2005) had already shown that most of the genes differentially expressed among Arabidopsis ecotypes were related to transcription and stress response, and indeed plants undergo significant transcriptional changes upon stress perception, with ABA specifically affecting ~10% of the Arabidopsis genome (Kreps et al., 2002; Song et al., 2016).

The improvement of sequencing technologies in the past decade has not only led to in-depth studies of natural variation in plant stress responses at the transcriptome level (Clauw et al., 2015; Clauw et al., 2016), but also allowed the global analysis of alternative splicing, where substantial natural variation has also been found, most prominently in stress-responsive genes (Gan et al., 2011; Chen et al., 2018b; Wang et al., 2018d; Khokhar et al., 2019b). Given the emerging role of post-transcriptional control in ABA signalling, ecotypes differing in ABA sensitivity would be expected to show, in addition to transcriptional differences, variations in their splicing response to the phytohormone.

Here, we identified the Arabidopsis natural variant Kn-0 as less sensitive to ABA during plant growth and development than the widely studied Col-0 ecotype. In agreement, a global transcriptome analysis revealed fewer and weaker changes in the molecular response to ABA for Kn-0 than Col-0, both at the gene expression and the alternative splicing level. Moreover, Kn-0 showed lower levels of core ABA-signalling SnRK2 protein kinases, a strong reduction in the expression of a key gene in ABA reactivation, and decreased basal ABA levels. Functional assessment of differential ABA-responsive splicing revealed alternative splicing events in key ABA pathway regulators that may underlie the distinct hormone sensitivity of the two ecotypes. Our results substantiate the involvement of alternative splicing in the implementation of ABA responses, while providing molecular insight into the natural variance of the plant's response to the phytohormone.

4.3 Results

4.3.1 The Arabidopsis accession Kn-0 is less sensitive to ABA than Col-0

Our preliminary physiological assays indicated that the Arabidopsis natural variant Kn-0, whose response to exogenous ABA had not to our knowledge been reported previously, differed in ABA sensitivity from the reference Col-0 ecotype. We therefore investigated differences in the responsiveness of these two Arabidopsis accessions to ABA in further detail by analysing different physiological and developmental processes affected by exogenous application of the phytohormone.

We first assessed seedling establishment by scoring cotyledon greening, a fundamental process in the transition to autotrophic growth, and found that, although almost all seeds of both natural variants germinated readily and fully developed into healthy seedlings under control conditions, the inhibitory effect of ABA on seedling greening was significantly less pronounced in the Kn-0 accession (Figure 4.1a). Indeed, 2 μM ABA reduced the proportion of green cotyledons in Kn-0 seedlings by less than 10%, while the same concentration of ABA inhibited the greening of Col-0 cotyledons by nearly 30% (Figure 4.1a).

We next turned to root growth and evaluated the response of the two accessions upon seedling transfer to ABA-containing medium. Col-0 and Kn-0 roots responded to the exposure to 2.5 μM ABA by inhibiting their primary root growth to a similar extent, with no statistical differences being detected between the two natural variants in either control or ABA conditions (Figure S4.1a). However, Kn-0 displayed a higher number of lateral roots than the Col-0 ecotype in both the absence and the presence of exogenous ABA, with the inhibitory effect of the phytohormone on the number of lateral roots being significantly less pronounced in Kn-0 (Figure S4.1b). As a result, the density of lateral roots was higher in Kn-0 under both control and ABA conditions, with the ABA inhibition of root branching being again significantly less severe in the Kn-0 accession (Figure 4.1b).

Finally, we used microscopy analyses to compare stomatal apertures in the two genotypes under control and ABA conditions (Figure 4.1c). In agreement with the reduced sensitivity to the phytohormone during cotyledon greening and root branching (see Figure 4.1a, b), we found that Kn-0 stomata were also significantly less responsive (~20%) to 10 μM ABA than those of Col-0 (Figure 4.1c).

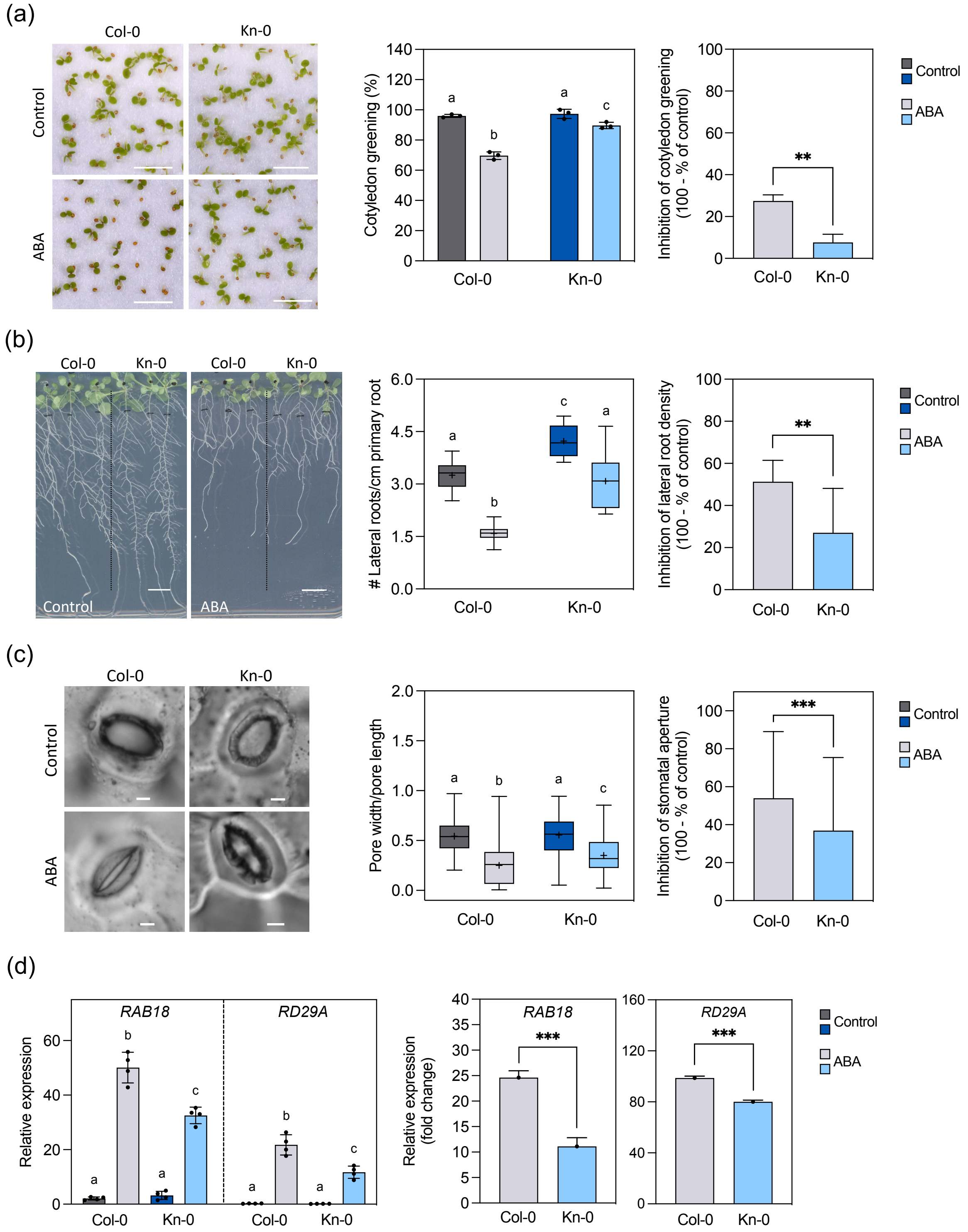

Figure 4.1. ABA phenotypes of *Arabidopsis thaliana* Col-0 and Kn-0 natural accessions.

(a) Seedling greening. Representative images of Col-0 and Kn-0 seedlings germinated under control conditions for 25 h and transferred to either control medium or 2 µM ABA for 48 h (scale bars = 0.5 cm), as well as quantification (means ± SD, *n* = 3) of the percentages of cotyledon greening and the respective inhibitory effect of ABA (100 - % of control conditions) in the same seedlings 27 h after transfer to control or ABA media. Results are representative of 8 independent experiments. Letters and asterisks denote statistically significant differences, with $p < 0.05$ (two-way ANOVA with Tukey HSD test) and $p < 0.01$ (Welch's *t*-test), respectively.

(b) Lateral root density. Representative images (scale bars = 1 cm) as well as quantification of lateral root density (number of lateral roots per cm of primary root) and the respective inhibitory effect of ABA (100 - % of control conditions) 11 d after transfer of 6-d-old Col-0 and Kn-0 seedlings to control conditions or 2.5 µM ABA. Data ($n = 10$) are represented as boxplots showing median (horizontal line), mean (+), interquartile range (IQR, 25-75 percentiles) and whiskers (highest and lowest values). Results are representative of 7 independent experiments. Letters and asterisks denote statistically significant differences, with $p < 0.05$ (two-way ANOVA with Tukey HSD test) and $p < 0.01$ (Welch's *t*-test), respectively.

(c) Stomatal aperture. Representative images (scale bars = 5 µm) of stomata from 9-d-old Col-0 and Kn-0 seedlings after 2 h in a stomatal opening solution followed by a 2-h control or 10 µM ABA treatment, as well as quantification of stomatal apertures (pore width/pore length) and the respective inhibitory effect of ABA (100 - % of control conditions) in the same seedlings. Data (n = 150-250) are represented as boxplots showing median (horizontal line), mean (+), interquartile range (IQR, 25-75 percentiles) and whiskers (highest and lowest values). Results are representative of 5 independent experiments. Letters and asterisks denote statistically significant differences, with $p < 0.05$ (Kruskal-Wallis test with Dunn's correction) and $p < 0.001$ (Welch's *t*-test), respectively.

(d) ABA marker gene expression. RT-qPCR analysis of the transcript levels and fold change of the *RAB18* and *RD29A* genes (means ± SD, $n = 4$) in Col-0 and Kn-0 seedlings grown on control medium for 4 d followed by a 3-h control or 5 µM ABA treatment. *PP2AA3* was used as a reference gene. Results are representative of 4 independent experiments. Letters and asterisks denote statistically significant differences, with $p < 0.05$ (two-way ANOVA with Tukey HSD test) and $p < 0.001$ (Welch's *t*-test), respectively.

To verify that the natural variance observed in the physiological response to ABA was substantiated at the molecular level, we selected two classical ABA marker genes, *RESPONSIVE TO ABA 18 (RAB18)* and *RESPONSIVE TO DESICCATION 29A (RD29A)*, and determined their expression levels in Col-0 and Kn-0 seedlings after a transient control or ABA treatment. As seen in Figure 4.1d, exposure to 5 *µ*M ABA induced the expression of both marker genes in the two accessions, but to a significantly lower extent (~50% and ~20% less induction for *RAB18* and *RD29A*, respectively) in Kn-0 compared with the Col-0 ecotype.

Together, the above results identify the Arabidopsis Kn-0 accession as less sensitive to ABA-induced stress than Col-0 across different physiological responses at different stages of early plant development, with the ABA hyposensitivity displayed by Kn-0 being corroborated at the molecular level.

4.3.2 ABA affects the expression of fewer genes in Kn-0 compared with Col-0

To gain deeper molecular insight into the difference in ABA sensitivity between the Col-0 and Kn-0 Arabidopsis accessions, we conducted an RNA-sequencing (RNA-seq) experiment to analye global transcriptomic differences between the two genotypes under both control and ABA conditions. To this end, 4-day-old seedlings grown in control conditions were transiently exposed (3 hours) to either control or 5 μM ABA-containing medium. cDNA libraries were prepared from three biological replicates and run using the Illumina HiSeq 2500 instrument, retrieving ~81 million paired-end reads of 125 bp of length per sample. The Kn-0 genome differs from that of Col-0 in ~637,000 single nucleotide polymorphisms (SNPs) as well as in thousands of insertions and deletions, some of which have significant disruptive effects such as those affecting splice sites or introducing premature stop codons (Gan *et al.*, 2020). We therefore found it necessary to align the reads of each natural variant to its corresponding genome — TAIR10 v.50 for Col-0 and the genome assembled by Gan et al. (2011) for Kn-0 — thus obtaining above 91% of uniquely-mapped reads for each sample.

To identify differentially expressed genes (DEGs) in response to ABA in either Arabidopsis accession, we used the DESeq2 package (Love et al., 2014) applying a Log2(fold change) > 1 or < -1 cut-off with an adjusted *p*-value < 0.01. Our analyses revealed a ~26% higher number of DEGs in Col-0 (2048, versus 1624 in Kn-0), with most of the DEGs in both accessions (59 and 67% in Col-0 and Kn-0, respectively) being upregulated by the hormone (Figure 4.2a and Table S1). Building on these findings, we further explored the overlap in DEGs between the two natural variants and observed that, for Kn-0, 79% of both up- and downregulated genes were shared with Col-0, which also showed a larger proportion of genes significantly changed only in that ecotype, *i.e* those that passed the cut-off criteria only in Col-0 (Figure 4.2a). Noticeably, as seen in Figure 4.2a, the difference in the number of DEGs between the two ecotypes was markedly more evident for the downregulated (58% higher in Col-0) than for the upregulated (11% higher in Col-0) genes, with the former also presenting a higher percentage of genes significantly changed only in Col-0 (50% versus 28% for the upregulated), suggesting that the higher transcriptional ABA responsiveness of the Col-0 ecotype in terms of DEG number may be attributed to disparities in repressed genes.

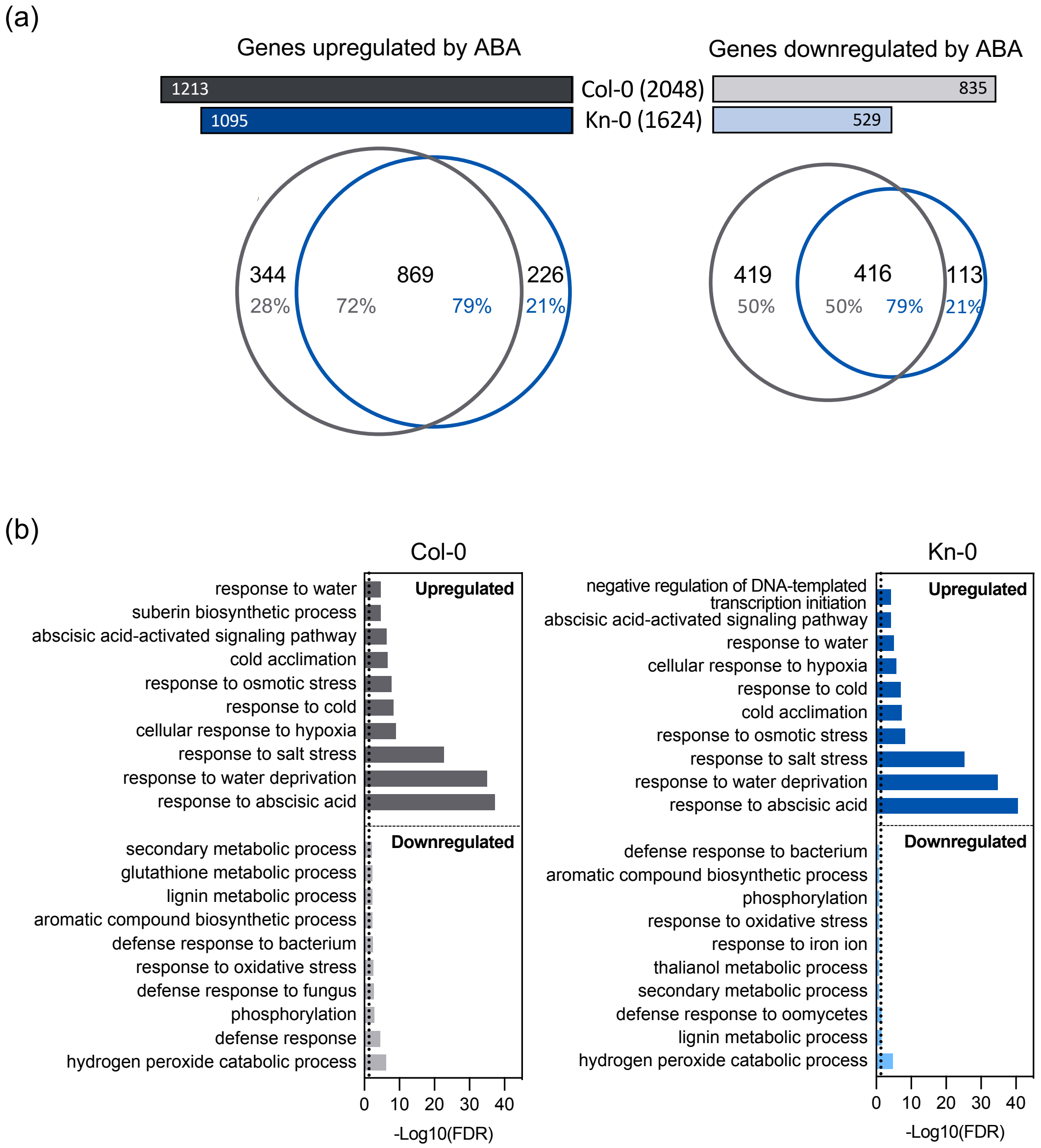

Figure 4.2. Genes regulated by ABA in the Arabidopsis Col-0 and Kn-0 accessions.

(a) Overlap between the genes up- (left) or down- (right) regulated by ABA in Col-0 and Kn-0. Values indicate the number of genes and corresponding percentages of the total up- or down-regulated in each accession. Graph bars indicate the number of up- and downregulated genes in Col-0 and Kn-0. The total number of DEGs is shown in parenthesis.

(b) Gene ontology analysis presenting the 10 most enriched biological processes for the ABA up- or downregulated genes in Col-0 and Kn-0. Dotted lines indicate the statistical significance threshold (FDR < 0.05).

A Gene Ontology (GO) term enrichment analysis revealed that the functional roles of the genes up- and downregulated in response to the ABA treatment were strikingly similar for Col-0 and Kn-0 (Figure 4.2b). As expected, upregulated genes in both natural variants showed an enrichment in the “response to abscisic acid” category as well as in functions associated with the response to ABA-related stresses, such as drought, salt and cold (Figure 4.2b). On the other hand, functions related to oxidative stress and defence responses to biotic stress appeared overrepresented for the ABA downregulated genes (Figure 4.2b). Similar results were found when examining the functions of the shared DEGs (Figure S4.2a and Table S4.2). Genes whose expression was found to be significantly changed in only one of the ecotypes also belong to functional categories typical of an ABA response. Genes significantly repressed by ABA only in Col-0 were enriched in functions related to the response to pathogens, with 57 genes including the term ‘defence’ in their biological process category (Figure S4.2b and Table S4.3). The GO term ‘glutathione metabolic process’, which comprised nine genes, was also significantly enriched in the downregulated genes (Table S4.3). The genes significantly induced by ABA only in Col-0 included 33, 15, and 14 loci with functions related to transcription, the response to abscisic acid — including genes encoding ABA signalling kinase SnRK2.6/OST1 and ABA transporter ATP-BINDING CASSETTE G25 (ABCG25) — and the response to salt, respectively, although their enrichment was not statistically significant (Figure S4.2b and Table S4.3). Regarding the genes significantly changed only in Kn-0, due to the low number retrieved, only among the upregulated was the ‘response to salt stress’ term, containing 13 genes, found to be significantly enriched (Figure S4.2b and Table S4.3). Similarly to what was observed for the Col-0 ecotype, genes significantly upregulated by ABA only in Kn-0 included 20 loci involved in transcriptional regulation and 9 in the response to ABA, while the ABA-repressed genes included 7 genes with functions related to transcription and 7 related to defence responses (Figure S4.2b and Table S4.3).

Thus, our analysis of the DEGs in response to ABA in the two natural accessions suggests that the reduced sensitivity of Kn-0 to the hormone when compared with Col-0 does not derive from the regulation of genes with distinct functions. Instead, it is likely attributable to other factors, such as a larger number of ABA-responsive genes in the Col-0 ecotype.

4.3.3 Gene expression levels are less affected by ABA in Kn-0 than in Col-0

As described above, most of the ABA-regulated genes were found to be common to Col-0 and Kn-0. However, the expression changes of these genes in response to ABA could differ significantly between the two ecotypes.

We first asked whether there would be genes oppositely regulated by ABA in Col-0 and Kn-0 — *i.e.*, upregulated in one accession while downregulated in the other or vice-versa — and identified five gene loci, all of which were repressed in Col-0 and induced in Kn-0. Of these, only one displayed notable (> 1 TPM in either all control or all ABA replicates) expression levels (Table S4.1): *NAC DOMAIN CONTAINING PROTEIN 3 (NAC003)*, a transcription factor with reported functions in ABA-related processes such as dark-induced senescence or vascular development (Chou et al., 2018; Yang et al., 2020; Hsu et al., 2022).

We then examined the fold-change values of the shared DEGs and found that, for both up- and downregulated genes, the changes in expression imposed by ABA were of lower magnitude in Kn-0 (Figure 4.3a), corroborating the ABA phenotypes observed at the physiological level (see Figure 4.1). The difference in fold change between the two ecotypes was more evident for the repressed genes (Figure 4.3a), again suggesting a larger impact of these genes on ecotype ABA sensitivity.

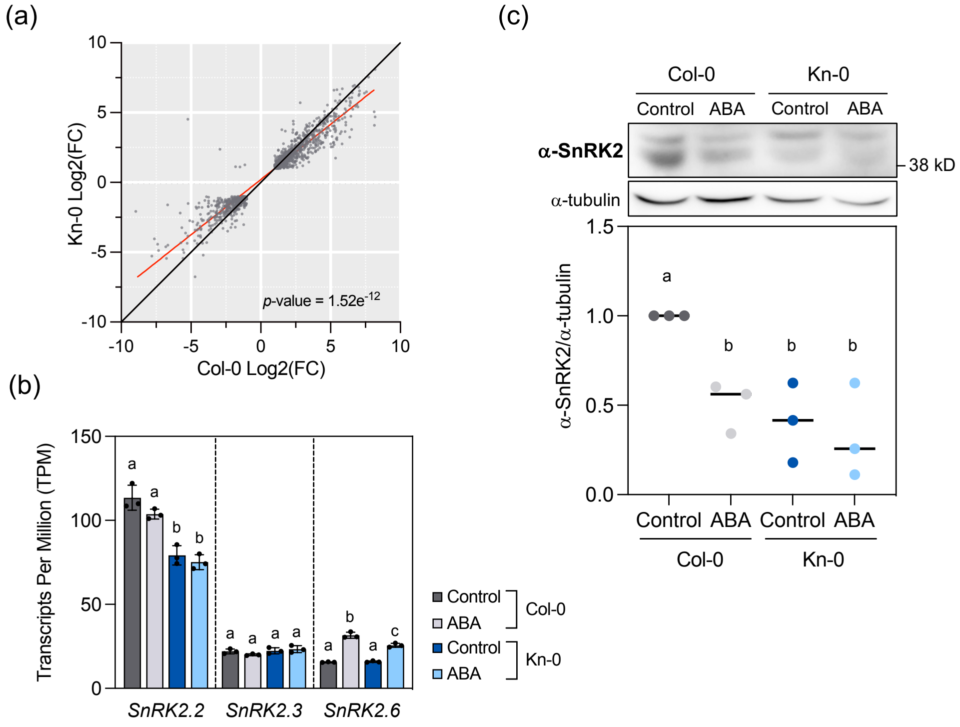

Figure 4.3. Expression of shared ABA-regulated genes and SnRK2 kinases in the Col-0 and Kn-0 accessions.

(a) Scatter plot of the Log2-fold changes in gene expression for the 1,290 genes regulated by ABA in both Col-0 and Kn-0. The p-value (ANCOVA F-statistics test) indicates statistically significant differences between the linear regression line for our data (red line) and the line representing equal Log2(FC) values for Col-0 and Kn-0 (black line).

(b) Expression levels (means ± SD, $n = 3$) of the Arabidopsis genes encoding subclass III SnRK2 kinases. Letters denote statistically significant differences, with $p < 0.05$ (two-way ANOVA with Tukey HSD test).

(c) Western blot analysis of SnRK2 protein levels in 4-d-old Col-0 and Kn-0 seedlings treated with 5 μM ABA for 3 h. Control samples were treated with the equivalent volume of the solvent of the ABA solution (ethanol). A total of 40 ng of protein was loaded per sample and a-tubulin antibodies were used as a loading control. Bands were quantified and relative protein levels determined using tubulin as a reference, with control conditions in the Col-0 accession set to 1. Results are from 3 biological replicates, and letters denote statistically significant differences with $p < 0.05$ (two-way ANOVA).

To investigate whether the reduced Kn-0 ABA sensitivity could also be attributed to differences in the expression of key components of ABA signalling, we compared the expression values of the genes encoding *i*) the PYL/PYR/RCAR family of intracellular ABA receptors, *ii*) clade A PP2C phosphatases, which bind to the receptors upon ABA perception, thus derepressing *iii*) SnRK2 subclass III kinases, which phosphorylate downstream effectors of the ABA pathway and are strongly activated by the hormone. Figure S4.3 shows that for genes encoding receptors and phosphatases involved in ABA signal transduction — which are in general repressed and induced by ABA, respectively — no significant differences were detected between the two Arabidopsis accessions (see also Table S4.4).

Importantly, regarding SnRK2 kinases, which are core signalling components of the ABA pathway, *SnRK2.2* was found to be constitutively ~30% less expressed in Kn-0 (Figure 4.3b and Table S4.4). This points to reduced ABA signalling in this accession compared with Col-0 and is consistent with the lower magnitude of change in the expression of both ABA up- and downregulated genes in Kn-0 (see Figure 4.3a). *SnRK2.6* also showed significantly lower expression levels in Kn-0 but solely upon ABA treatment, while *SnRK2.3* exhibited similar expression values in both conditions and ecotypes (Figure 4.3b and Table S4.4). To determine whether the differences in *SnRK2* transcript levels were mirrored at the protein level, we used western blotting to quantify the amount of SnRK2 kinases present in the two accessions under both control and ABA conditions. In line with the mRNA analyses (Figure 4.3b), Kn-0 seedlings contained lower levels of SnRK2 proteins than Col-0 under non-stress conditions (Figure 4.3c). Exposure to ABA reduced significantly SnRK2 protein amounts in Col-0, with no significant differences observed between ecotypes under ABA conditions, despite a noticeable trend towards lower levels in Kn-0 (Figure 4.3c).

To examine how natural variation could be influencing SnRK2.2 levels, we used the Polymorph1001 tool (https://tools.1001genomes.org/polymorph/) to identify sequence variants between Col-0 and Kn-0 (Table S4.11). None of the polymorphisms found in the coding sequence result in missense variants (Table S4.11), pointing to no impact on protein function. However, 10 of the 11 polymorphisms found in the *SnRK2.2* promoter are located within transcription factor binding sites (TFBS), as identified by the PlanPAN4.0

(http://plantpan.itps.ncku.edu.tw/plantpan4/promoter_analysis.php) analysis tool (Table S4.12). These sites are predicted to interact with specific transcription factors, including members of the AP2/ERF, bZIP, MYB or NAC families, which play crucial roles in regulating stress responses (Song et al., 2016). Any variations in these sites could affect transcription factor binding affinity and in turn *SnRK2.2* transcriptional activation, providing an explanation for the observed differences between the two ecotypes.

In sum, the reduced ABA sensitivity of the Kn-0 accession compared with Col-0 may also arise from a lower magnitude of ABA-induced changes in gene expression. Furthermore, it could stem from decreased levels of key ABA signalling components, such as the core SnRK2 kinases, possibly due to genetic polymorphisms in the promoter sequence.

4.3.4 Kn-0 has a lower endogenous ABA content than Col-0

Reduced ABA sensitivity and signalling in the Kn-0 accession would be consistent with lower basal endogenous levels of the hormone. We therefore measured ABA in Col-0 and Kn-0 seedlings grown under control conditions. We indeed found decreased ABA levels in Kn-0 (Figure 4.4), in agreement with both smaller changes in ABA-regulated gene expression and lower SnRK2 protein levels under non-stress conditions (see Figure 4.3). To corroborate this finding at the physiological level, we assessed seed dormancy, which depends primarily on endogenous ABA content, in the two natural accessions. As seen in Figure S4.4, germination rates of freshly-harvested, non-stratified seeds were higher for Kn-0, indicating reduced seed dormancy levels, as expected for the ecotype with lower ABA content.

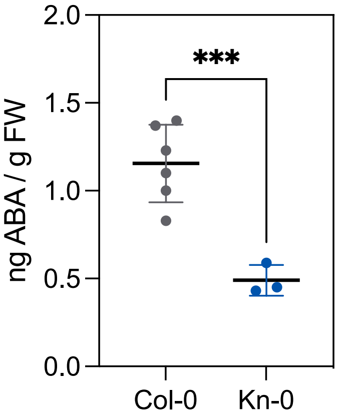

Figure 4.4. Endogenous ABA levels in Col-0 and Kn-0.

ABA concentrations (means ± SD, n = 3-6), determined by mass spectrometry, of 4-d-old Col-0 and Kn-0 seedlings grown under control conditions. Asterisks denote a statistically significant difference, with $p < 0.001$ (Welch's *t*-test).

Given the differences in basal ABA levels, we next looked closely at the expression of the main genes involved in the hormone's biosynthesis, catabolism, and glycosylation. Up to the final plastidial step in the ABA biosynthetic pathway, all genes involved were ~20-60% more expressed in Kn-0, both in the absence and the presence of exogenous ABA (Figure S4.5 and Table S4.5). An exception was *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3)*, which encodes the key rate-limiting enzyme in ABA biosynthesis, whose expression under control conditions was 57% higher in Kn-0, but no substantial difference was observed in the presence of ABA (Figure S4.5 and Table S4.5). As for the last step in ABA biosynthesis, *MOLYBDENUM COFACTOR SULFURASE* (*ABA3*) transcript levels were unchanged both by the ABA treatment and between the ecotypes (Figure S4.5 and Table S4.5). As for the ABA catabolic pathway, under both conditions the *CYTOCHROME P450 FAMILY 707 SUBFAMILY A (CYP707A)* genes either showed no significant differences between the two natural variants or were more expressed in Kn-0. These data suggested an elevated ABA production in Kn-0 compared with Col-0 under control conditions, being in apparent conflict with the lower ABA content found in Kn-0. However, lower transcript levels of the genes responsible for reverting the glycosylated inactive form of ABA, ABA-glucose ester (ABA-GE), to its active state (ABA) were detected in Kn-0, especially of *ß-GLUCOSIDASE 1 (BG1*) whose expression is severely reduced (~80%) in Kn-0 under both control and ABA conditions (Figure S4.5 and Table S4.5).

As conducted for *SnRK2.2*, we next investigated whether genetic polymorphism could underlie the lower *BG1* transcript levels in Kn-0. We identified six sequence variants in the promoter region (Table S4.11), all of which located within predicted TFBS associated with transcription factor families implicated in stress responses (Table S4.12), such as AP2/ERF, bZIP, MYB, or NAC (Song et al., 2016), and hence potential contributors to the difference in *BG1* RNA levels between the two ecotypes. In addition, several sequence differences were identified in the gene's coding region, three of which result in missense variants (p.266N>K; p.289L>I; p.365S>N), all within the glycosyl hydrolase family 1 (PF00232) domain, potentially leading to altered protein functions (Tables S4.11).

Together, these findings indicate that the Kn-0 ecotype has constitutively lower endogenous ABA levels than Col-0. Moreover, despite elevated expression of ABA biosynthesis genes, a key gene in the reactivation of stored ABA reserves is markedly downregulated in the Kn-0 ecotype, possibly due to sequence variations in its promoter.

4.3.5 ABA induces less alternative splicing changes in Kn-0 than in Col-0

Accumulating evidence of the involvement of alternative splicing in plant stress responses raised the question of whether, as found with gene expression, the splicing response to ABA would be weakened in the ABA hyposensitive Kn-0 ecotype. To address this, we used rMATS-turbo (Shen et al., 2014) to detect alternative splicing events induced by ABA in the same samples analysed for gene expression. Differential alternative splicing (DAS) was considered when an event exhibited a difference in absolute inclusion levels (*i.e.*, the proportion of reads that include the alternative sequence) higher than 0.1 between control and ABA treatments. Alternative splicing events were classified into four major types: exon skipping (ES), intron retention (IR), alternative 5' splice site (A5SS) and alternative 3' splice site (A3SS).

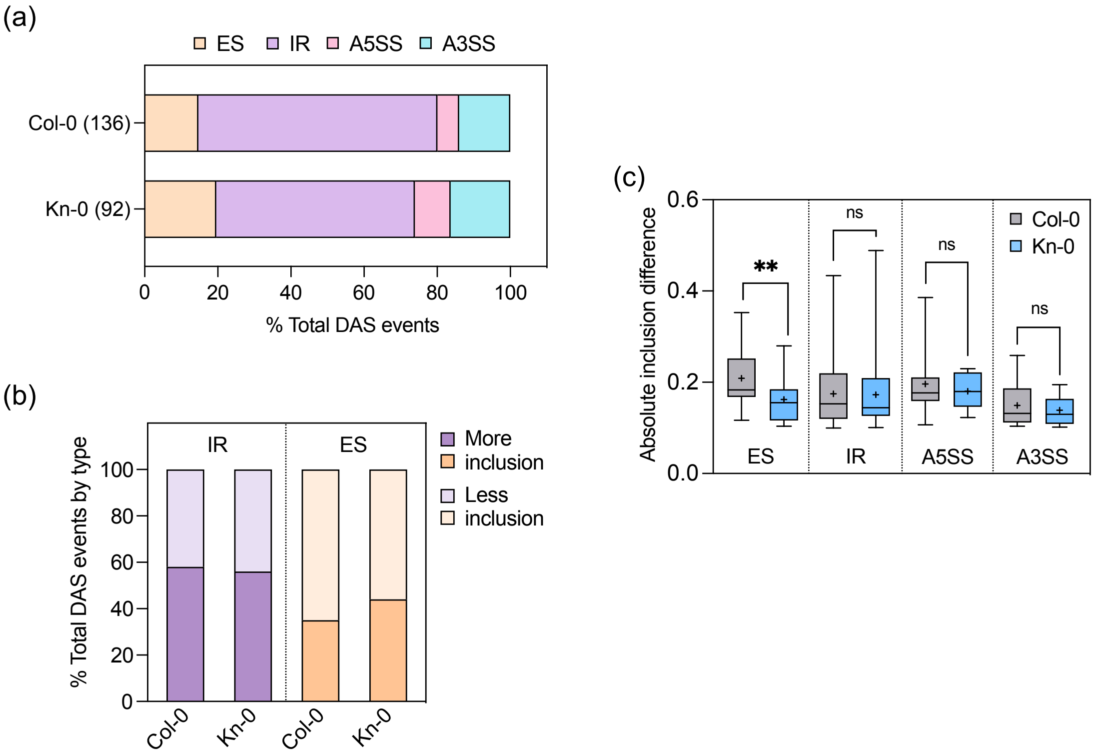

Figure 4.5. Alternative splicing events regulated by ABA in Col-0 and Kn-0.

(a) Contribution of the major differential alternative splicing (DAS) event types to the global ABA-induced alternative splicing response in Col-0 and Kn-0. The total number of DAS events is indicated in parentheses.

(b) Percentage of IR and ES events for which the alternatively-spliced sequence is more or less included by ABA in Col-0 and Kn-0.

(c) Changes in absolute inclusion levels for each of the major types of alternative splicing events responding to ABA in Col-0 and Kn-0. Data [*n* (Col-0/Kn-0) = 18/20 (ES), 50/89 (IR), 8/9 (A5SS), 15/19

(A3SS)] are represented as boxplots showing the median (horizontal line), mean (+), interquartile range (IQR, 25-75 percentiles) and whiskers (highest and lowest values). Asterisks denote statistically significant differences, with $p < 0.01$ (Mann-Whitney test). Exon skipping, ES; Intron retention, IR; Alternative 5' splice site, A5SS; Alternative 3' splice site, A3SS.

In line with the differences found in gene expression levels, analysis of the data revealed ~32% fewer alternative splicing events responding to ABA in the Kn-0 accession: 92 DAS versus 136 in Col-0 (Figure 4.5a and Table S4.6 and S4.7). The distribution of alternative splicing event types was similar in the two ecotypes, with the most common, comprising 54-65% of the total DAS events, being IR (Figure 4.5a and Table S4.6 and S4.7), which is the most prevalent type in plants (Ner-Gaon et al., 2004; Filichkin et al., 2010; Marquez et al., 2012b; Martín et al., 2021). However, we did find a notable difference for ES events, with ABA inducing less exon skipping (Figure 4.5b) and a significantly lower magnitude of change (Figure 4.5c) in Kn-0 compared to Col-0.

Finally, detected changes in alternative splicing were validated with a 100% success rate via an alternative method. For all of twelve randomly selected events (six per ecotype), RT-PCR using primers specifically designed to flank the alternative sequence yielded very similar results to those obtained in the RNA-seq splicing analysis (Figure S4.6 and Table S4.8).

These results show that in both the Col-0 and Kn-0 accessions splicing patterns are changed in response to ABA, with the most affected alternative splicing event type being IR. However, consistent with reduced ABA sensitivity at the physiological level, Kn-0 shows fewer changes in alternative splicing compared to Col-0. The proportion of skipped exons upon ABA exposure and the magnitude of the splicing change for ES events are also reduced in the Kn-0 ecotype.

4.3.6 ABA changes alternative splicing of a distinct set of genes in Col-0 and Kn-0

We next examined the genes affected in their splicing pattern by ABA (DAS genes). As some genes include more than one ABA-responsive alternative splicing event, the number of DAS genes is lower than the number of DAS events, and the difference between ecotypes for the former was also lower than for the latter — as shown in Figure 4.6, Kn-0 displayed only ~27% fewer DAS genes than Col-0 (76 versus 104), whereas it had shown ~32% fewer DAS events (see Figure 4.5a).

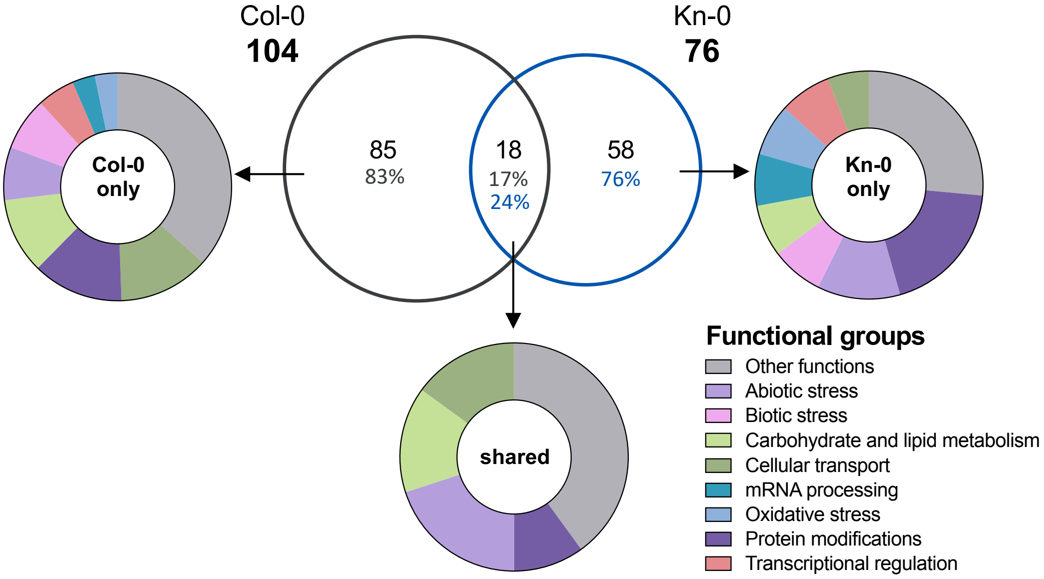

Figure 4.6. Genes differentially spliced in response to ABA in Col-0 and Kn-0.

Overlap of DAS genes (genes containing DAS events) between the Col-0 and Kn-0 accessions. Values indicate the number of genes and corresponding percentages of the total DAS genes in each accession. Pie charts indicate the proportion of each functional group for the shared DAS genes and those significantly changed in only one of the ecotypes.

To investigate whether the ABA splicing response targeted the same loci or genes fulfilling similar functions in both ecotypes, we overlapped the lists of their DAS genes before conducting GO analyses. Only 18 DAS genes were shared between the two ecotypes (17% and 24% of the total DAS in Col-0 and Kn-0, respectively), most were significantly changed only in one of the ecotypes (Figure 4.6 and Table S4.9). A GO analysis of all DAS genes for each ecotype did not reveal significantly-enriched terms, but we nonetheless grouped terms with related GO-terms and looked at the weight of each of these functional groups for the shared DAS genes and those whose splicing was only significantly changed in one of the ecotypes (Figure 4.6).

DAS genes shared between Col-0 and Kn-0 were found to be involved in a wide variety of functions. Out of the 18, four encode proteins previously linked to ABA responses: RGLG1, an E3 ubiquitin ligase that targets PP2CA phosphatases for degradation (Wu *et al.*, 2016); ERD15, a negative regulator of ABA signalling (Aalto *et al.*, 2012); ALDH7B4, which scavenges toxic aldehydes and reactive oxygen species (ROS) produced upon stress (Kotchoni et al., 2006); and a putative kinase (AT1G18390/LRK10L1) whose mRNA variant produces a plasma membrane protein involved in ABA signalling (Lim et al., 2015) (Table S4.9). DAS events affecting three of these genes occur within the 5'UTR, suggesting roles in regulating mRNA translation or stability. Events in *RGLG1* and *ALDH7B4* exhibited similar

ABA-induced changes in inclusion levels, while differences between Col-0 and Kn-0 were observed for *ERD15* (Table 4.1). The ES event in *LRK10L1* affects the coding sequence, with ABA promoting exon inclusion and potentially altering protein function to different extents in the two ecotypes (Table 4.1).

Table 4.1. ABA-induced changes in selected DAS events.

Gene ID	Gene name	AS event type	AS event region	InclDiff Col-0	InclDiff Kn-0
AT3G24170	*GR1*	ES	5'UTR	-0.171 -0.153	-0.004 -0.035
AT3G01650	*RGLG1*	ES	5'UTR	0.254	0.261
AT1G54100	*ALDH7B4*	IR	5'UTR	0.265	0.255
AT2G41430	*ERD15*	IR	5'UTR	0.252	0.149
		IR	5'UTR	0.301	0.156
AT1G18390	*LRK10L1*	ES	CDS	0.269	0.164
AT2G48120	*PAC*	IR	CDS	0.002 -0.007	0.210
AT1G67080	*ABA4*	IR	CDS	0.002	0.151
		A5SS	CDS	-0.006	0.230

AS, alternative splicing; InclDiff, inclusion difference; ES, exon skipping; IR, intron retention; A5SS, alternative 5' splice site; 5'UTR, 5' untranslated region; CDS, coding sequence.

Regarding the DAS genes significantly changed in only one ecotype, the most abundant functions identified for Col-0 relate to transport processes, protein modifications and carbohydrate or lipid metabolism, while among the DAS genes changed significantly only in Kn-0 the most frequent categories were protein modifications and response to abiotic stress (Figure 4.6 and Table S4.6 and S4.7). The genes whose splicing was only changed significantly in one of the ecotypes were also involved in biotic stress, mRNA processing, oxidative stress, and transcriptional regulation processes and included 14 with a demonstrated role in regulating the ABA pathway (Figure 4.6 and Table S4.9). For example, in *GR1*, which encodes an antioxidant enzyme, we identified an alternative exon in the 5'UTR whose inclusion was changed by ABA only in Col-0 (Table 4.1). On the other hand, *PAC* and *ABA4*, involved in carotenoid and ABA biosynthesis, respectively, undergo alternative splicing events in the coding sequence that were only ABA-responsive in Kn-0 (Table 4.1), again pointing to a differential contribution to ABA responses.

Overall, ABA-mediated changes in alternative splicing affected a lower number of genes in the Kn-0 ecotype. Both the DAS genes shared between Col-0 and Kn-0 and those exhibiting significant changes in only one ecotype include established key regulators of the ABA response. Among these, we identified several ABA-induced alternative splicing events that may underlie different ecotype ABA sensitivity.

4.3.7 Alternative splicing provides a more ecotype-specific regulatory layer of the ABA response than transcription

Previous reports have indicated an independent role for transcriptional and splicing regulation during plant stress responses (Ding et al., 2014; Calixto et al., 2018; Chen et al., 2018b; Martín et al., 2021). Notably, when we assessed the overlap between genes with ABA-induced changes in gene expression and in splicing, only 12 or 14% of the genes differentially spliced in response to ABA (DAS genes) were also regulated at the gene expression level by ABA in Kn-0 (9 out of 76) or Col-0 (15 out of 104), respectively (Figure 4.7 and Table S4.10). While this observation suggests that transcription and splicing may operate independently in regulating the ABA response, it is important to note that these two processes can also influence each other.

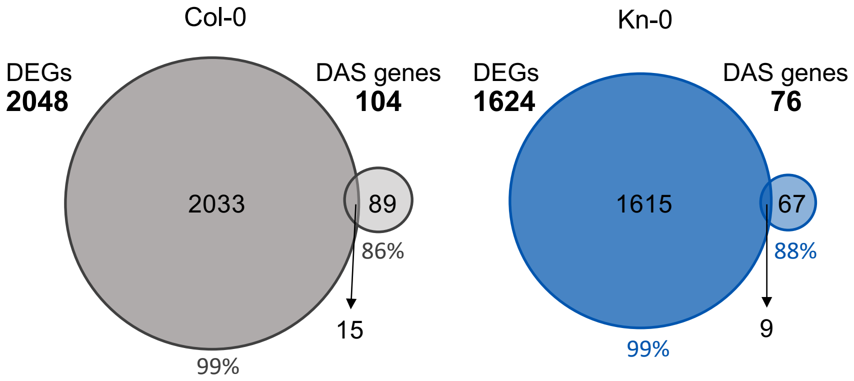

Figure 4.7. Genes differentially expressed or spliced in response to ABA in Col-0 and Kn-0.

Overlap of the genes ABA regulated at the gene expression or splicing levels in the Col-0 and Kn-0 natural accessions. For each accession, values indicate the number of genes and corresponding percentages of the total DEGs or DAS genes.

Of the loci regulated by ABA both at the expression and splicing level, we identified three that were common to the two Arabidopsis natural accessions: *ALDH7B4*, encoding aldehyde dehydrogenase 7B4, *GPDHC1,* encoding 6-phosphogluconate dehydrogenase family protein, and AT5g01670, encoding a NAD(P)-linked oxidoreductase superfamily protein.

Interestingly, as seen in Figures 4.2c and 4.6, the proportion of genes shared between the two ecotypes is very different for the DEGs and the DAS genes. Among the genes for which splicing was affected by ABA, only 17-24% were common to the two ecotypes (see Figure 4.6), while these percentages raised to 63-79% (1285 out of 2048 or 1624 for Col-0 or Kn-0, respectively) for the DEGs (see Figure 4.2a). This suggests that alternative splicing plays a more important role in determining the differential ABA sensitivity of Col-0 and Kn-0 than gene expression levels.

The above results point to the alternative splicing process as a more ecotype-specific mode of regulation of the ABA response than transcription, and suggest that the two processes represent, at least in part, independent regulatory layers, as previously suggested in the literature.

4.4 Discussion

The phytohormone ABA is a pivotal integrator of abiotic stress signals that enables plants to cope with environmental changes by tightly regulating and coordinating different molecular responses. In Arabidopsis, exogenous application of ABA is well known to lead to extensive changes in transcript levels (Kreps et al., 2002; Song et al., 2016) and has also been found to affect alternative splicing, promoting the usage of non-canonical splice sites and targeting mainly genes with functions that are complementary to those of genes altered in expression (Zhu et al., 2017b). On the other hand, the expression of splicing factors was shown to be affected in both ABA-deficient and ABA-signalling mutants (Cruz et al., 2014). Moreover, genetic sequence variations in Arabidopsis are known to influence the expression and splicing of several genes, including stress-responsive genes, and could contribute to differences in ABA content and the response to environmental stress among Arabidopsis accessions (Barrero et al., 2006; Gan et al., 2011; Kalladan et al., 2017; Wang et al., 2018d). In line with emerging evidence for post-transcriptional control of ABA signalling, we hypothesized that plant natural variance in ABA sensitivity would correlate not only with differences in ABA-responsive gene expression but also in the splicing response to the hormone.

Preliminary assays conducted on several Arabidopsis natural variants had indicated that Col-0 and Kn-0 seedlings responded differently to ABA. We thus subjected these two ecotypes to treatments of different duration and ABA concentrations, consistently observing in Kn-0 a weaker physiological response to the hormone than Col-0, from the early stages of seedling establishment to seedling root growth or stomatal movements. We then selected 4-day-old seedlings and a 3-h 5 μM ABA treatment for a global transcriptomic comparison of the Col-0 and Kn-0 accessions to evaluate the early molecular response to ABA.

While our transcript level analyses did not reveal significant differences between Col-0 and Kn-0 for genes encoding ABA intracellular receptors or PP2Cs under control or ABA conditions, this was not the case for *SnRK2.2* or *SnRK2.6*, two of three subclass III SnRK2 kinases of the ABA signalling pathway. SnRK2.6, which is primarily found in guard cells and controls ABA-induced stomatal closure (Postiglione and Muday, 2020), was equally expressed in both natural variants under control conditions but less expressed in Kn-0 upon ABA exposure, indicating reduced activation of the pathway in response to stress. On the other hand, *SnRK2.2*, which transduces the ABA signal ubiquitously and is mainly involved in seed germination, seedling growth and gene expression regulation (Fujii et al., 2007), was constitutively less expressed in Kn-0. This could stem from polymorphisms found in the gene's promoter sequence that are likely to affect binding of transcription factors implicated in ABA-mediated stress responses, such as AP2/ERF, bZIP, MYB or NAC family members (Song et al., 2016). At the protein level, Kn-0 presented lower amounts of SnRK2 kinases in the absence of stress. Given that the *snrk2.2* single mutant displays ABA insensitivity phenotypes (Fujita et al., 2009), this may explain why Kn-0 is less responsive to ABA. The decline in protein levels upon 3 hours of ABA exposure, particularly in Col-0, could result from shutting down of the system in response to the treatment (Ali et al., 2020; Wang et al., 2020).

Analysis of the endogenous ABA content of the two Arabidopsis natural variants revealed lower basal levels in Kn-0, which could underlie its enhanced lateral root growth under control conditions when compared with Col-0. This is consistent with the inhibitory effect of ABA on lateral root growth, resulting in overall larger root systems when ABA synthesis or signalling is defective (Harris, 2015). Unexpectedly, under both control and ABA conditions, key genes in the ABA biosynthetic pathway were generally expressed at higher levels in Kn-0. Exceptions were *NCED3*, encoding the key rate-limiting enzyme, which showed comparable expression levels to Col-0 upon ABA exposure, and the unchanged transcript levels of *ABA3*, involved in the last biosynthetic conversion step, that could result in equivalent ABA production rates in Col-0 and Kn-0. A more active biosynthetic pathway in Kn-0 is in apparent contradiction with its lower ABA content. However, we found that *BG1,* a key gene in the release of ABA from the inactive ABA-GE (Lee et al., 2006), is substantially less expressed in Kn-0. Conjugated forms of hormones are used by plants to quickly respond to stress before activating *de novo* biosynthesis (Kollist et al., 2019). In line with the reduced ABA responsiveness of Kn-0, loss of *BG1* function causes ABA-deficient phenotypes such as impaired stomatal closure, faster seed germination, reduced ABA content and abiotic-stress sensitive phenotypes (Lee et al., 2006). Moreover, upregulation of the biosynthetic pathway in Kn-0 could reflect a compensatory mechanism for the reduced production of active ABA via ABA-GE hydrolysis, consistent with the hypothesis of crosstalk between these two different routes of ABA production put forward by Xu et al. (2012). Interestingly, we identified multiple variations in the sequence of *BG1* between the two ecotypes that may explain the lower *BG1* transcript levels in Kn-0 and hence the differences in ABA sensitivity. Further in-depth polymorphism analyses could provide valuable insight into the role of natural genetic diversity in ABA responses.

Globally, our analysis of the RNA-seq data revealed a reduction of ~21% and ~32% in the number of differentially expressed and differentially spliced genes, respectively, clearly showing that ABA induced fewer changes not only in gene expression but also in alternative splicing in the least ABA-sensitive accession, Kn-0. Transcriptomic studies comparing natural variants with opposite stress sensitivities have been essentially conducted in other plant species and with inconsistent results — genotypes more sensitive to an osmotic stressor, such as salt, do not always change more their expression and splicing patterns in response to stress than tolerant cultivars (Shankar et al., 2016; Li et al., 2018b; Mirdar Mansuri et al., 2019; Kaundal et al., 2021; Kong et al., 2021; Zhang et al., 2021a; Ye et al., 2022). Nonetheless, these studies largely agree on the importance of the stress-tolerant variant activating genes with relevant stress functions.

As expected, our ABA treatment led to significant gene expression changes. Unsurprisingly, induced genes in both Arabidopsis accessions were involved mainly in ABA-related and stress-responsive functions, falling in categories such as “response to abscisic acid” or “response to water deprivation”. On the other hand, genes downregulated by ABA were enriched in functions related to oxidative stress and defence responses, namely genes associated with the catabolism of hydrogen peroxide (H_2O_2), a key ROS stress signalling molecule (Huang et al., 2019a). The enrichment in defence functions could stem from indirect ABA-mediated repression. While ABA has been primarily associated with abiotic stress, it is also involved in plant-pathogen interactions in a context-dependent manner: the hormone enhances initial defences by closing stomata and promoting callose accumulation to prevent pathogen entry, but once inside the plant (Hewage et al., 2020), ABA signalling activation negatively regulates other hormonal pathways involved in biotic stress responses, rendering the plant more susceptible to infection (Berens et al., 2017). Notably, the difference between Col-0 and Kn-0 was more pronounced for the ABA-downregulated genes, indicating that repression of oxidative stress and defence functions may contribute more to the differential ABA response of the two ecotypes.

Given that both natural variants responded, albeit to a different extent, to the ABA treatment at the physiological level, we expected to observe common molecular changes. Indeed, our results show a shared hormonal response where 80% of the expression changes in Kn-0, mainly in genes involved in ABA and stress-related responses, overlapped with those of Col-0, despite the lower magnitude of change consistent with reduced ABA sensitivity. Genes found significantly changed in only one of the ecotypes are also likely to contribute to the differential response to the hormone. Genes significantly upregulated by ABA only in Col-0 included those involved in transcription and ABA-mediated stress responses, while downregulated genes were enriched in defence responses and in glutathione transferases (GST), which aid in ROS detoxification, enhancing stress tolerance (Chen et al., 2012). As for the genes induced by ABA only in Kn-0, we found an enrichment in salt stress responses, consistent with previous studies indicating higher salt tolerance for Kn-0 when compared to Col-0 (Quesada et al., 2002; Katori et al., 2010). The increased Kn-0 germination rates under salt stress shown in Quesada et al. (2002) are in line with its lower ABA content. Conversely, the reduced survival of Col-0 under salt stress reported by Katori et al. (2010) may result from the adverse effects of prolonged elevated ABA levels, which could impair photosynthesis, deplete energy or reduce growth (Wang et al., 2018b). The differences in salt tolerance between ecotypes could also be attributed to ABA-independent pathways. In agreement with lower ABA sensitivity when compared to Col-0, Kn-0 displays a weaker root hydrotropic response, which was found to depend on ABA signalling (Miao et al., 2021).

Besides gene expression, ABA and abiotic stress in general are also known to affect mRNA splicing. Consistent with previous reports (Ding et al., 2014; Calixto et al., 2018; Chen et al., 2018b; Martín et al., 2021), we found a minimal overlap between genes with changes at the expression and alternative splicing levels, which has been interpreted as transcription and splicing acting as two distinct regulatory layers in the response to stress. However, it is crucial to recognize the interplay between these processes. Alternative splicing has been shown to influence transcription by modulating transcript stability, notably through mechanisms such as the nonsense-mediated decay (Kalyna et al., 2012). Conversely, transcription can impact alternative splicing outcomes by regulating the expression of splicing factors or by affecting splice site recognition through modulation of the speed of transcription (Braunschweig *et al.*, 2014; Ullah *et al.*, 2023). Nevertheless, the very low number of shared targets regulated at both the transcription and splicing levels alongside the clear ecotype specificity of the ABA splicing response compared to gene expression (~20% versus ~80% overlap) supports the notion that these mechanisms are at least partially independent in the ABA response. In fact, only three genes were regulated by ABA at both levels in the two natural variants: *GPDHC1*, whose loss of function causes delayed germination and seedling establishment under ABA conditions as well as constitutively higher ROS levels (Shen et al., 2006); *ALDH7B4*, whose knockout mutants are more sensitive to abiotic stresses and also accumulate more ROS (Kotchoni et al., 2006); and *AT5G01670*, found to be part of the *ABI3* regulon (Mönke et al., 2012). Their ABA-related functions and common regulation point to these three genes as central players in both the transcriptional and post-transcriptional layers of regulation of the ABA response.

Among the ABA-related genes undergoing DAS events in both ecotypes, *RGLG1* and *ALDH7B4* show similar ABA-induced splicing changes and could represent important drivers of the ABA response, while DAS events in *ERD15* and *LRK10L1* are likely contributors to the differential ABA sensitivity of Col-0 and Kn-0. *ERD15*, a reported negative regulator of the ABA response (Aalto et al., 2012), shows higher levels of intron retention in its 5' UTR upon ABA treatment in Col-0, which could contribute to differential sensitivity of the two ecotypes. On the other hand, inclusion levels of an ES event in the coding sequence of *LRK10L1* are higher in Col-0, in line with the ecotype's higher responsiveness to ABA. In fact, inclusion of this alternative exon promotes expression of a *LRK10L1* transcript whose loss of function causes insensitivity to the hormone during early plant development (Lim et al., 2015).

Of the key ABA components among the splicing targets retrieved in only one of the ecotypes, two positive regulators were found to be particularly interesting, *PAC* and *ABA4*. *PAC* is involved in chloroplast development, has been linked to biosynthesis of carotenoids, which are ABA precursors, and mutants lacking *PAC* are ABA-deficient (Holding et al., 2000). An ABA-induced IR event affecting the *PAC* coding sequence occurs only in Kn-0 and introduces a premature stop codon, which could either target the transcript to degradation via the NMD pathway or result in the production of a truncated protein, thus leading to reduced ABA content and sensitivity. Similarly, *ABA4* positively regulates ABA biosynthesis and its loss of function leads to reduced ABA content (Dall'Osto et al., 2007; North et al., 2007). Two DAS events located in the *ABA4* coding sequence and predicted to downregulate gene expression were induced by ABA exclusively in Kn-0: an IR event, introducing a premature stop codon, and an A5SS event, resulting in the production of a shorter protein without altering the reading frame. The above examples provide clear functional links between differential splicing and ABA sensitivity.

Finally, our results suggest a prevalent role for ES events in regulating the ABA response. Plant exposure to abiotic stress has been found to lead to more skipping of alternative exons

(Martín et al., 2021). In line with this, we observed that the less ABA sensitive Kn-0 accession responded to the hormone by skipping fewer alternative exons than Col-0. Furthermore, the proportion of ES events was higher in Kn-0 and these displayed a lower magnitude of change in response to ABA when compared with Col-0.

Our study identifies two Arabidopsis natural variants, Col-0 and Kn-0, as differing in their physiological and molecular responses to ABA. We show that Kn-0 is less sensitive to ABA, which correlates with reduced gene expression and alternative splicing changes upon exposure to the hormone. While a strong common response is observed at the gene expression level, both genes responding to ABA in only one of the ecotypes and a specific splicing response are also likely to determine the differential ABA sensitivity of the two accessions. Our findings also suggest that reduced expression, likely due to natural sequence variations, of key ABA signalling and reactivation components contributes to the lower sensitivity of Kn-0 to ABA. Importantly, analysis of ABA-responsive alternative splicing changes in the two ecotypes identified functionally-relevant differences in established ABA pathway regulators that likely contribute to the ABA-deficiency and hyposensitivity of Kn-0. Although the significance of alternative splicing and its coordination with transcription to ensure stress responses tailored to the plant's environmental context is yet to be fully understood, our results corroborate a crucial role, complementary to transcription, for alternative splicing in implementing the ABA response and offer new molecular insight into the inherent natural variation of the plant's response to the hormone.

4.5 Experimental procedures

4.5.1 Plant material, growth conditions and phenotypical assays

The *Arabidopsis thaliana* Col-0 (N70000) and Kn-0 (N76969) accessions were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Prior to any experiment, plants were grown in parallel for two generations in growth chambers under long-day photoperiod: 16 h light/8 h dark at 21-24°C (100-120 μmol m^{-2} s^{-1} light intensity)/18°C (dark) and 60-65% relative humidity (RH). For phenotypical assays, unless otherwise specified, seeds were surface-sterilized with a bleach-based solution [20% (v/v) bleach, 0.25% (v/v) sodium dodecyl sulfate (SDS)] for 10 min, plated on MS medium [1 x MS basal salt mix, 0.01% (w/v) myo-inositol, 0.05% (w/v) MES, pH 5.7, 0.8% (w/v) agar], and stratified for 3 days at 4°C in the dark before transfer of the plates to a growth cabinet under long-day conditions (22°C light 30% RH/18 °C dark 70% RH, 100 μmol m^{-2} s^{-1} light intensity) or continuous light (22°C, 40% RH, 93 μmol m^{-2} s^{-1} light intensity).

To assess dormancy, seeds were surface-sterilized as above 2 days after harvest, sown without prior stratification on MS medium plates and incubated under continuous light, with radicle emergence being scored after 18, 24, 48 and 72 h in the light. Cotyledon greening assays were conducted according to Albuquerque-Martins et al (2022): seeds were stratified for 4 days and germinated on filter paper in control medium plates for 25 h in continuous light before transfer to either control medium or 2 μM ABA (Duchefa Biochemie) for 48 h. Root assays were performed as described previously by Remy and Duque (2016): after stratification for 3 days, seeds were germinated vertically in control medium plates under long-day conditions for 5 days and then transferred to control or 2.5 μM ABA plates for 11 days. Stomatal aperture assays were based on Díez et al. (2022): seeds were stratified for 3 days and grown in control medium plates under long-day conditions for 9 days, before transfer to a stomatal-opening solution for 2 h followed by a 2-h control or 10-μM ABA treatment in liquid MS medium, with cotyledon impressions being then obtained with dental resin and imaged under a bright-field microscope.

Primary root length and stomatal apertures were determined using the Fiji software (Schindelin et al., 2012). All statistical analyses were conducted with GraphPad Prism (v. 9.1.1).

4.5.2 Sample preparation and RNA sequencing

Col-0 and Kn-0 seeds were surface-sterilized and stratified at 4°C for 65 h on filter paper in control medium plates before transfer to continuous light conditions. After 4 days, seedlings were transferred to control or 5-μM ABA plates for 3 h. Three biological replicates per ecotype and treatment were collected and RNA extracted from the plant material using the innuPREP Plant RNA kit (Analytik Jena). Pre- and post-sequencing RNA quality control using a fragment analyser (Advanced Analytical Technologies, Inc), preparation of cDNA libraries from polyA-selected mRNA, and sequencing using the HiSeq Sequencing V4 Chemistry kit (Illumina, Inc) and HiSeq 2500 sequencer (Illumina, Inc), which generated ~81 million paired-ends 125 bp reads per sample analysed with the HiSeq Control Software 2.2.58 (Illumina, Inc), were all carried out at the Centre for Genomic Regulation (Barcelona, Spain).

4.5.3 Read alignment, transcriptome reconstruction and mapping

For the Col-0 ecotype, the TAIR10 genome and the annotation from the Ensembl release 50 were used, while for Kn-0 unmasked genome and consolidated annotation (*de novo* integrated with TAIR10 annotations) files were retrieved from http://mtweb.cs.ucl.ac.uk/mus/www/19genomes/19genomes.htm, the Gan et al. 2011 reference-based assembly part of the 1001 Genomes Project.

The Gffread tool (https://github.com/gpertea/gffread) was used to convert GFF3 into GTF files using the -T option. Genome indexes were generated from the FASTA genome sequences and the GTF annotation files using the following parameters in STAR (Dobin et al., 2013): --runMode genomeGenerate --sjdbOverhang 124 --genomeSAindexNbases 12 --runThreadN 8. RNA-seq reads were then mapped to each of the Arabidopsis ecotypes' genome using the splice-aware aligner STAR (Dobin et al., 2013) to generate the alignment BAM files using the options --outSAMtype BAM SortedByCoordinate --sjdbOverhang 124 --alignIntronMax 500000 --runThreadN 8. For all samples of both ecotypes, more than 91% of the reads were uniquely mapped to the corresponding genome.

4.5.4 Quantification of global changes in gene expression

The featureCounts function of the Rsubread package (Liao et al., 2014; Liao et al., 2019) was used to generate gene count lists from the BAM and GTF files using the options nthreads = 8, isPairedEnd = TRUE, isGTFAnnotationFile = TRUE. Differential gene expression analysis was conducted using the DESeq2 software (Love et al., 2014) from raw counts using independentFiltering=TRUE. Genes were considered differentially expressed if Log2(fold change [ABA vs control]) > 1 / < -1 with an adjusted *p*-value < 0.01. Comparison of Log2(FC) between the two ecotypes was conducted using linear regression, with the *p*-value being calculated from an ANCOVA F-statistics test using GraphPad Prism (v. 9.1.1). Counts normalized to library size retrieved from DESeq2 using the function counts and the argument normalized = TRUE were used to calculate transcripts per million (TPM) at the gene level with an R script available from http://ny-shao.name/2016/11/18/a-short-script-to-calculate-rpkm-and-tpm-from-featurecounts-output.html. Calculation of TPM considers gene length (of key importance to compare expression levels between two ecotypes that contain many sequence variants), which is not taken into account in the DESeq2 count normalization. Genes were included in the TPM analysis only if TPM counts were greater than 1 in either all control sample replicates or all ABA sample replicates.

4.5.5 Quantification of global changes in alternative splicing

The rMATS-turbo pipeline (Shen et al., 2014) was used to detect and quantify alternative splicing event inclusion levels (proportion of reads containing the alternative sequence), ranging from 0 to 1, from junction counts (*i.e.* considering only reads that span splice junctions), using the arguments -t paired --readLength 125 --nthread 8 --novelSS. The latter option allows for the discovery of novel, unannotated splice sites as long as one end of the splice junction is annotated, by adjusting the other end with information from the BAM and GTF files. To quantify differential alternative splicing (DAS) between conditions, we filtered out events displaying < 10 average inclusion and skipping junction reads in both treatments and considered an event to undergo DAS if the difference in inclusion levels between the two treatments was > 0.1 or < -0.1, with an adjusted *p*-value < 0.05 (likelihood-ratio test with the Benjamini & Hochberg adjustment). For the subsequent analysis, only the four main types of AS events were considered: intron retention (IR), exon skipping (ES), alternative 5' splice sites (A5SS) and 3' (A3SS) splice sites.

4.5.6 Functional enrichment and overlap analyses

To retrieve Gene Ontology (GO) terms and perform GO enrichment analysis, the DAVID Knowledgebase v2021q4 (Huang et al., 2009a; Huang et al., 2009b) was used. For enrichment analysis of biological processes (GOTERM_BP_DIRECT), thresholds of a false discovery rate (FDR) < 0.05 and a minimum of 2 gene counts were applied to consider a term significantly enriched. Background sets for the functional enrichment analyses were defined — to analyse all Col-0 and Kn-0 DEGs, all genes for which DESeq2 retrieved an adjusted *p*-value were used, while for the DEGs common to both ecotypes the backgrounds of the two natural variants were unified. For a global analysis of the functions of the few DAS genes retrieved (Fig. 4.6), the biological process terms obtained from DAVID (GOTERM_BP_DIRECT) were grouped into broader functional categories.

Overlapping of datasets was conducted in Microsoft Excel (v. 16.59) and proportional Venn diagrams were built using the VennDiagram R package (Chen, 2021).

4.5.7 Polymorphism Analysis

Sequence variation was analysed using the 1001 Genomes Project Polymorph1001 tool (https://tools.1001genomes.org/polymorph/). Transcription factor binding sites (TFBSs) were predicted using the PlanPAN4.0 site Promoter Analysis tool (http://plantpan.itps.ncku.edu.tw/plantpan4/promoter_analysis.php). The promoter region of each gene was determined by counting 1000bp upstream of the 5'UTR in the corresponding strand orientation, except when the intergenic region was smaller.

4.5.8 Analysis of the expression and alternative splicing of individual genes

The expression levels of the individual genes shown in Figure 4.1d were analysed by RT-qPCR using plant material grown under the same conditions as for the RNA-seq experiment. Total RNA, extracted as above, was subjected to Dnase treatment using the RQ1 Dnase kit (Promega), and cDNA was synthesized using the SuperScript III Reverse Transcriptase (Invitrogen) and an oligo(dT) primer. qPCR was performed in the Applied Biosystems

QuantStudio 7 platform with Luminaris Colour HiGreen qPCR Master Mix (Thermo Scientific). Relative quantification was assessed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and *PP2AA3* as a reference gene (Figure S4.7).

The validation of selected DAS events was conducted using cDNA from 3-4 biological replicates of 4-day-old seedlings subjected to 3-h control or ABA treatments. Plant growth conditions, treatments and cDNA synthesis were as described above. RT-PCR was performed using the NZYTaqII 2x Green Master Mix (NZYtech) and primers flanking the alternative sequence designed by Primer3 (Untergasser *et al.*, 2012). The following PCR program was used: 95ºC 3 min / 35 x (95ºC 30 s / 55ºC 30 s / 72 ºC 30 s) / 72ºC 5 min. Amplified products were run for 30-70 min on a 2% agarose gel and bands were quantified using the Fiji software (Schindelin et al., 2012).

The sequences of all primers used for RT-qPCR and RT-PCR analyses are listed in Table S4.8. Statistical analyses were conducted using GraphPad Prism (v. 9.1.1).

4.5.9 Protein extraction and quantification

Four-day-old seedlings, grown and subjected to same treatment as for the RNA-seq samples, were ground into a fine powder in liquid nitrogen with mortar and pestle. Total protein was extracted by addition of a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Triton™ X-100 (Sigma-Aldrich) supplemented with Complete Protease Inhibitor Cocktail (Roche). The extract was cleared from cell debris by centrifugation at 18,000 *g* for 10 min at 4°C. Protein amounts in the supernatant were determined using Bradford reagent (Bio-Rad) in a spectrophotometer measuring the absorbance at 595 nm using known dilutions of BSA (NEB) as a standard. Equal amounts of protein extracts were resolved on an 8% SDS-polyacrylamide gel after a denaturation step with 5X Laemmli buffer and 8% β-mercaptoethanol (95 °C for 5 min). The proteins were then transferred to PVDF membranes (Immobilon-P, Millipore) and blocked with 5% (w/v) non-fat dry milk in 1X Tris-buffered saline (TBS, 25 mM Tris-HCl pH 7.4, 137 mM NaCl) for 2 h. The membranes were probed overnight at 4°C with anti-SnRK2 antibodies (Agrisera,1:3,000 dilution) in 1% (w/v) non-fat dry milk, 1X TBS and then with peroxidase-conjugated anti-rabbit secondary antibody (Amersham, 1:5,000 dilution) for 2 h at room temperature. After each incubation with the antibodies, the membranes were washed with TBS containing 0.05% Tween® 20 (Sigma-Aldrich) for 30 min. As a loading control, the same membranes were probed with anti-tubulin antibody clone DM1A (Sigma, 1:4,000 dilution), followed by incubation with peroxidase-conjugated anti-mouse secondary antibody (Jackson Immunoresearch, 1:5,000 dilution). Specific bands were visualized by enhanced chemiluminescence using SuperSignal® West FEMTO Maximum Sensitivity Substrate (Thermo Scientific Pierce). The intensity of the protein bands was quantified using the Fiji software (Schindelin et al., 2012). Three independent experiments were performed, and statistical differences between samples were inferred by two-way ANOVA using GraphPad Prism (v.9.1.1).

4.5.10 Quantification of endogenous ABA levels

For ABA measurements, samples were collected from four-day-old seedlings of the Col-0 and Kn-0 ecotypes grown as described above for the RNA-seq samples. 50 mg of lyophilized tissue was ground and resuspended in 80% methanol—1% acetic acid solution containing internal standards (deuterium-labelled hormones; OlChemim Ltd., Olomouc, Czech Republic). Tissue collection and extractions were conducted in triplicate. The mix was shaken for one hour at 4 °C, and the extracted fraction was incubated overnight at −20 °C. Samples were centrifuged, and the supernatant was vacuum dried and dissolved in 1% acetic acid. A reverse-phase column (OasisHLB) was used, and the eluate was dried and dissolved in a solution of 5% acetonitrile and 1% acetic acid. An autosampler and reverse-phase UHPLC chromatography column, 2.6 µm Accucore RP-MS, 100 mm × 2.1 mm (Thermo Fisher Scientific, San Diego, CA, USA) was used. ABA was separated through a gradient of acetonitrile (2–55%) containing 0.05% acetic acid at a rate of 400 µL/min over 22 min and detected in a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific; San Diego, CA, USA). Targeted Selected Ion Monitoring and Electrospray Ionization in the negative mode were used to detect ABA. Measurements were performed using external calibration curves with the Xcalibur 4.0 and TraceFinder 4.1 SP1.

4.6 Data statement

All raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE2283.

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4.8 Author contributions

ARD and PD conceived the project and designed the research, ARD, DS and JL-J performed the experiments, and ARD and PD analysed the data and wrote the manuscript. All authors contributed to the interpretation of results, critically reviewed the manuscript, and approved its final version.

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4.10 Supporting information

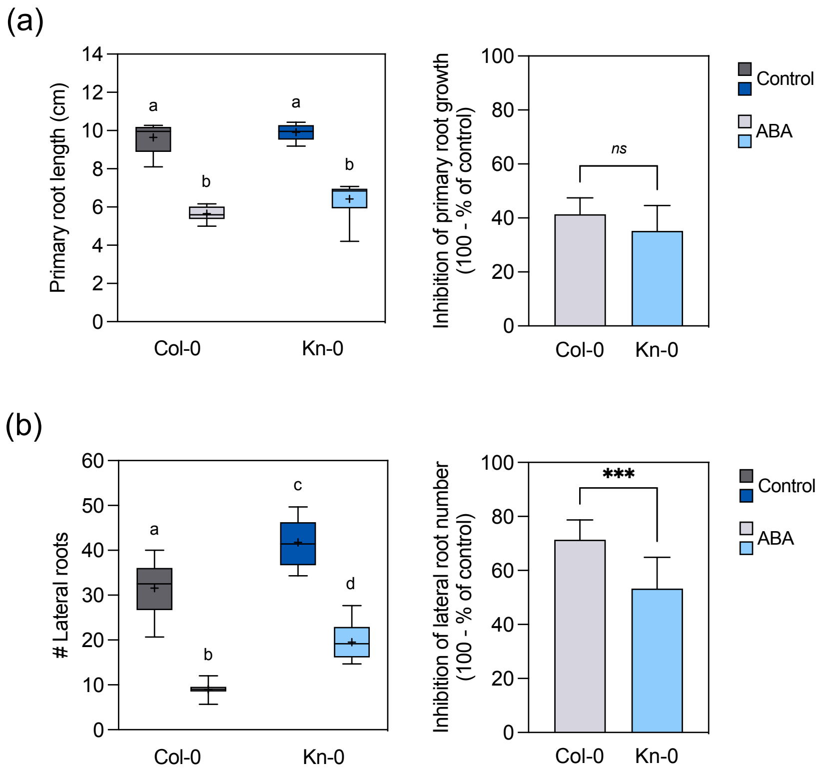

Figure S 4.1. Root ABA phenotypes of *Arabidopsis thaliana* Col-0 and Kn-0 natural accessions.

Primary root length (a) and number of lateral roots (b) as well as the respective inhibitory effects of ABA (100 - % of control conditions, means ± SD, *n* = 10) scored 11 d after transfer of 6-d-old Col-0 and Kn-0 seedlings to control conditions or 2.5 µM ABA. Data (*n* = 10) are represented as boxplots showing median (horizontal line), mean (+), interquartile range (IQR, 25-75 percentiles) and whiskers (highest and lowest values). Results are representative of 7 independent experiments. Letters denote statistically significant differences ($p < 0.05$) assessed using Kruskal-Wallis test with Dunn's correction (a) or two-way ANOVA with Tukey HSD test (b). Asterisks indicate statistically significant differences with $p < 0.001$ and *ns* no statistically significant differences with $p > 0.05$ (Welch's *t*-test).

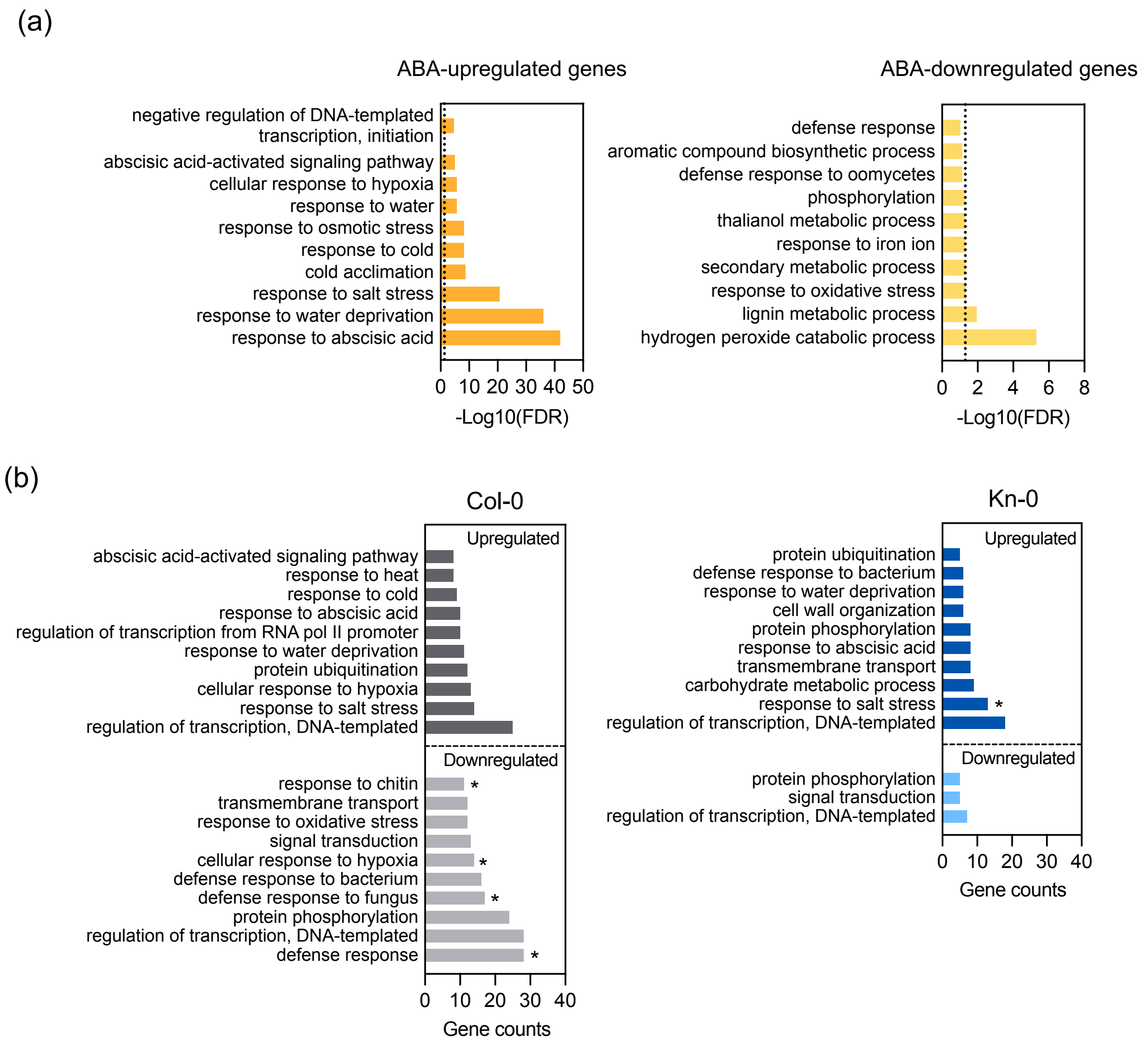

Figure S 4.2. Gene Ontology (GO) analysis of shared and of significantly ABA-regulated genes in only one of the ecotypes.

(a) The 10 most enriched biological process categories for the genes up- and downregulated by ABA in both Col-0 and Kn-0. Dotted lines denote statistically significance threshold (FDR < 0.05).

(b) The top 10 biological process categories, ordered by number of gene counts, for the genes up- and downregulated by ABA in either Col-0 or Kn-0. Only terms including 5 or more gene counts are shown. Asterisks denote GO-term enrichment with a false discovery rate (FDR) < 0.05.

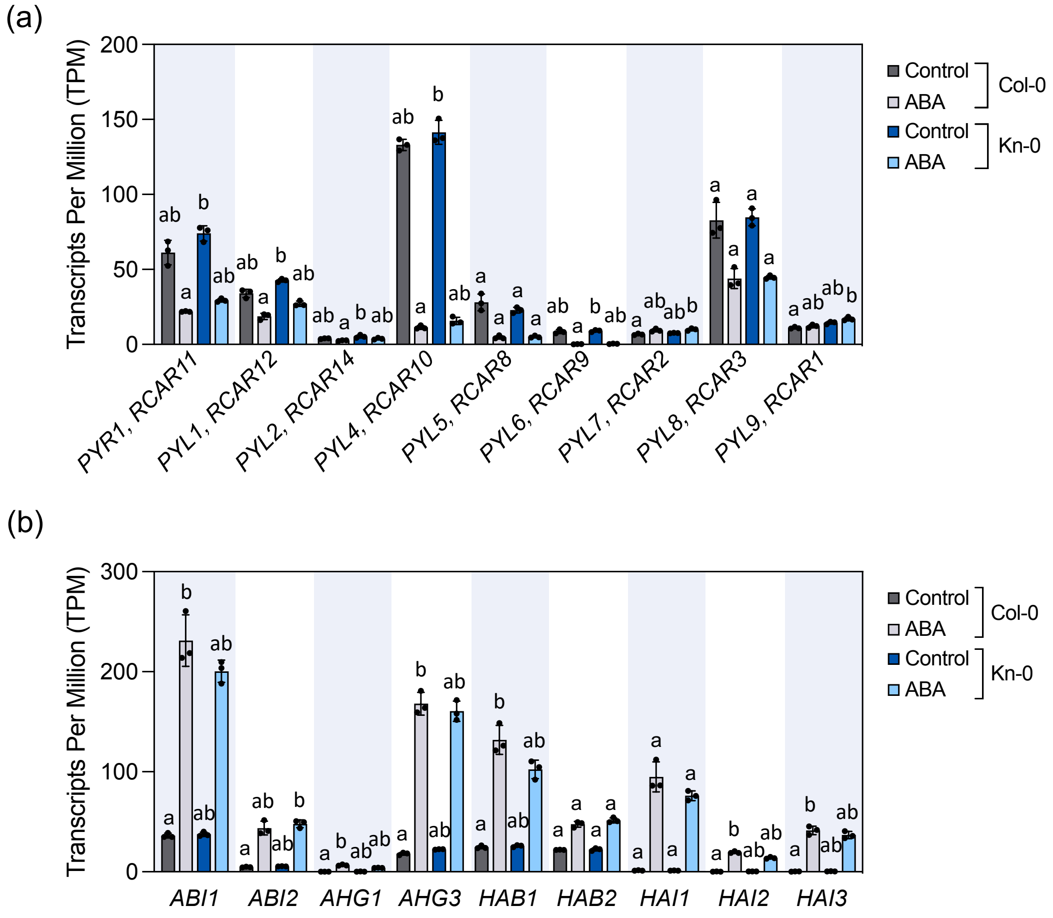

Figure S 4.3. Expression levels of genes encoding receptors and phosphatases of the ABA signalling pathway in the Arabidopsis Col-0 and Kn-0 accessions.

Gene expression levels (means ± SD, $n = 3$) of (a) PYR/PYL/RCAR receptors and (b) PP2C phosphatases. Letters denote statistically significant differences, with $p < 0.05$ (Kruskal-Wallis test with Dunn's correction).

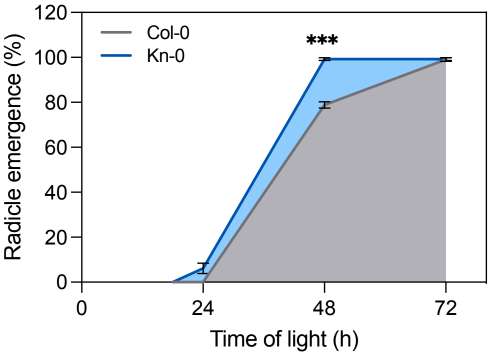

Figure S 4.4. Seed dormancy phenotype of the Col-0 and Kn-0 accessions.

Germination rates of freshly-harvested, non-stratified Col- 0 and Kn-0 seeds scored by assessing emergence of the radicle under control conditions during the first 3 d after transfer to light (means ± SD, *n* = 3). Asterisks denote statistically significant differences between the two natural accessions at each time point, with *p* < 0.001 (Welch's *t*- test).

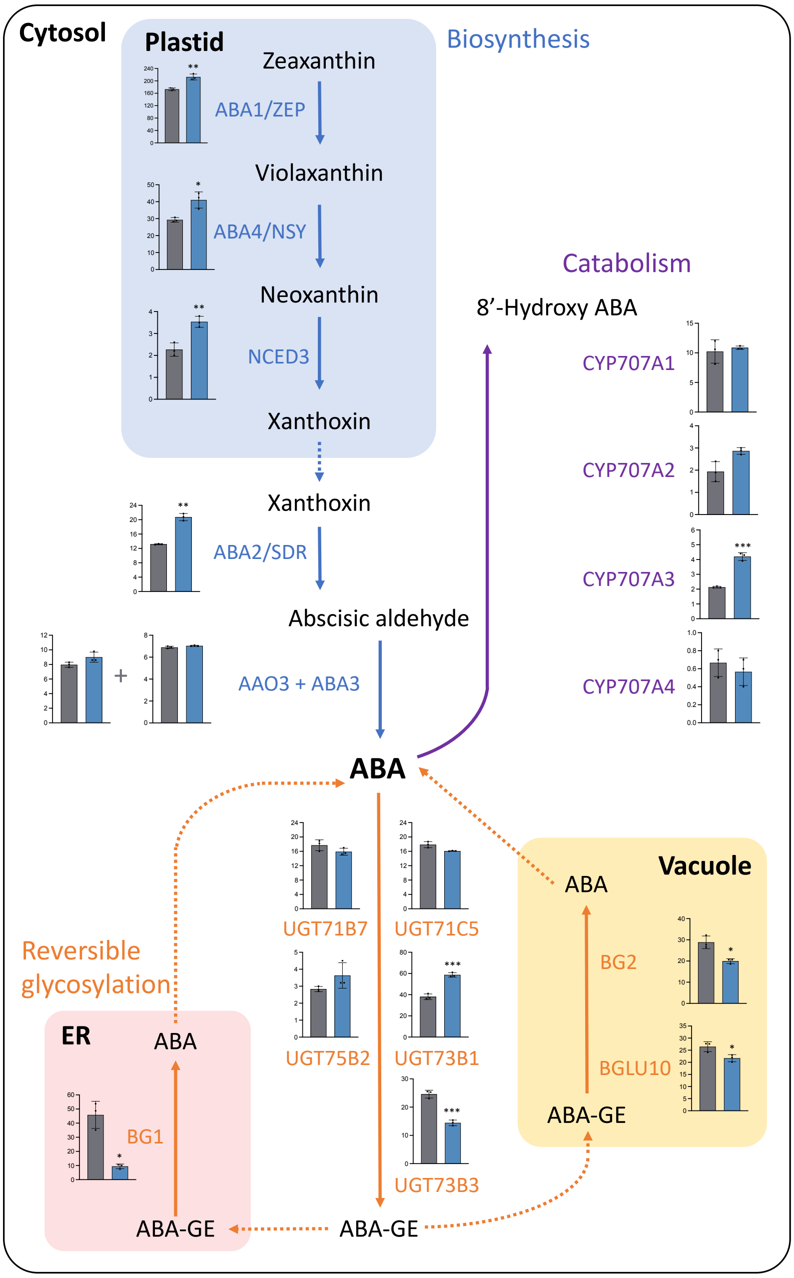

Figure S 4.5. Basal expression of ABA metabolism genes in Col-0 and Kn-0.

Basal (under control conditions) expression levels of genes involved in ABA metabolic pathways (biosynthesis in blue, catabolism in purple and reversible glycosylation in orange). Bar graphs indicate transcripts per million (TPM) values ($n = 3 \pm$ SD) obtained for the Col-0 (grey bars) and Kn-0 (blue bars) accessions. TPM values were calculated from DESeq2 normalized counts, and only genes with TPM > 1 in both accessions were considered. Dotted arrows indicate transport between different cellular compartments. Asterisks denote statistically significant differences between Col-0 and Kn-0, with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Welch's *t*-test).

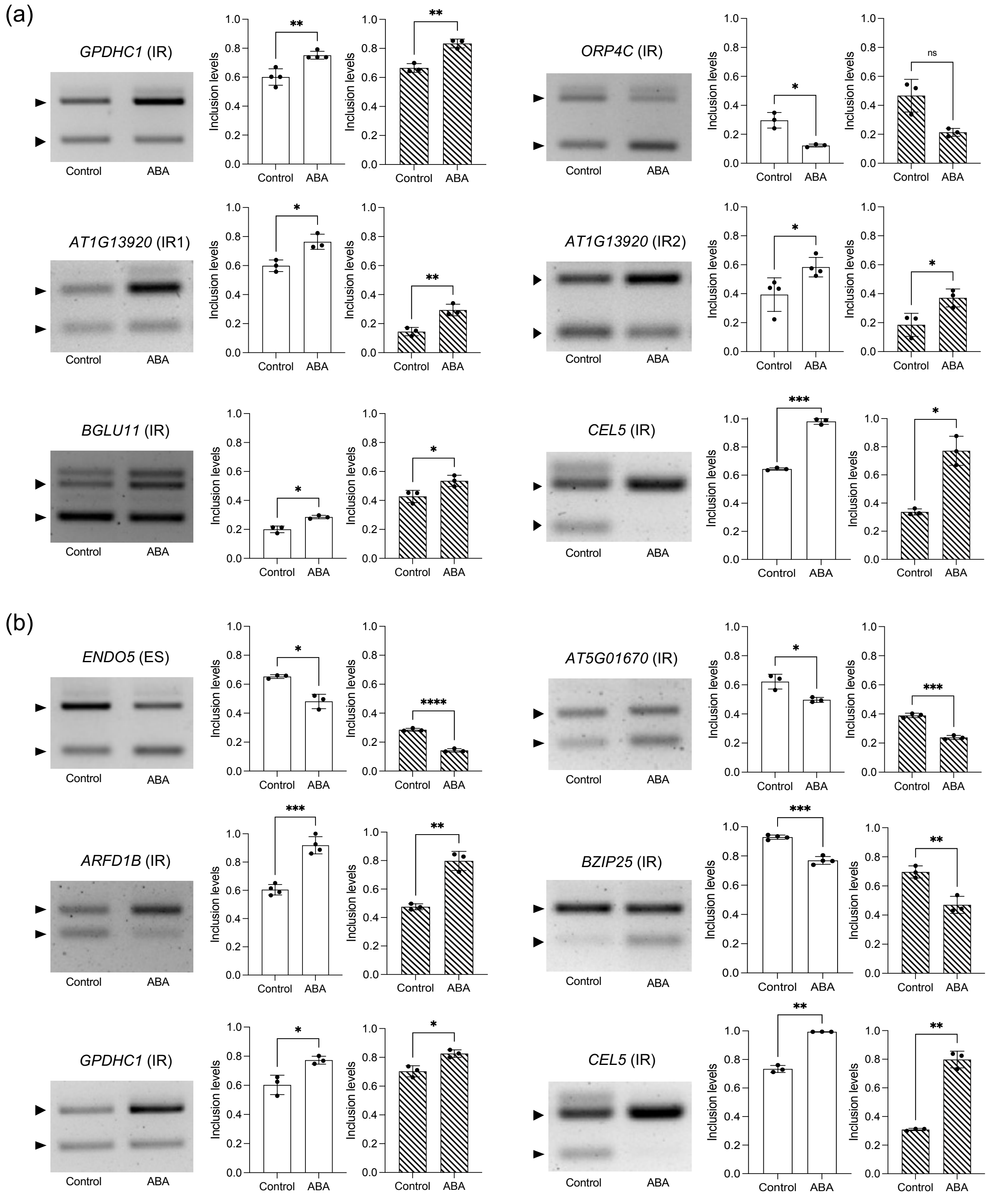

Figure S 4.6. Validation of selected differential alternative splicing (DAS) events identified by rMATS.

RT-PCR analysis of individual alternative splicing events differentially regulated upon a 3-h 5 µM ABA treatment in 4-d-old seedlings of the Col-0 (a) and Kn-0 (b) natural accessions. Graphs represent inclusion levels (means ± SD, *n* = 3-4) ranging from 0 (always excluded) to 1 (always included) after quantification either of the intensity of the relevant RT-PCR bands (arrowheads) using the Fiji software (white bars) or of RNA-seq data using rMATS (striped bars). Asterisks denote statistically significant differences between control and ABA conditions, with *p < 0.05, **p < 0.01, ***p < 0.001 (Welch's *t*-test). Intron retention, IR; Exon skipping, ES.

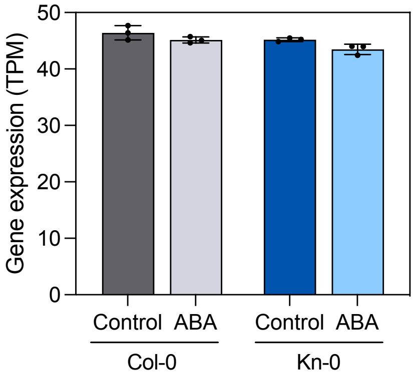

Figure S 4.7. Expression of the *PP2AA3* gene in Col-0 and Kn-0.

Expression levels (means ± SD, n = 3) calculated as TPM (Transcripts per Million) retrieved from DESeq2 counts. Within each ecotype, no statistically significant differences were observed between control and ABA conditions (two-way ANOVA).

Supplementary tables are available as Excel files at the following link: https://doi.org/10.1111/tpj.16773

Table S4.1. Differentially expressed genes (DEGs) in response to ABA in the Arabidopsis Col-0 and Kn-0 accessions.

Table S4.2. GO-term enrichment analysis of the ABA DEGs shared between Col-0 and Kn-0.

Table S4.3. GO-term enrichment analysis of the ABA DEGs retrieved in only one of the ecotypes.

Table S4.4. Expression levels of ABA signalling genes in Col-0 and Kn-0.

Table S4.5. Expression levels of ABA metabolism genes in Col-0 and Kn-0.

Table S4.6. Differential alternative splicing (DAS) events in response to ABA in Col-0.

Table S4.7. Differential alternative splicing (DAS) events in response to ABA in Kn-0.

Table S4.8. Primer sequences used in this study.

Table S4.9. Functional groups of DAS genes.

Table S4.10. Genes regulated by ABA at both the gene expression and splicing levels.

Table S4.11. *BG1* and *SnRK2.2* genetic variation between Col-0 and Kn-0.

Table S4.12. Predicted transcription factor binding sites affected by polymorphisms in the *BG1* and *SnRK2.2* promoters.

Chapter 5. General Discussion and Conclusions

Plants require robust mechanisms to survive stressful conditions and balance everyday fluctuations in growth environments. Abscisic acid (ABA), a crucial phytohormone, plays a major role in growth and stress responses, particularly in coordinating responses to abiotic stresses such as drought and high salinity. The ABA pathway is intricately regulated at multiple levels, providing a safety mechanism in case other regulators fail. It is well known that ABA affects the transcriptional landscape of plants. Moreover, alternative splicing – a mechanism enabling the production of different mRNA isoforms from the same gene – is being increasingly recognized as a key player in the regulation of plant responses to environmental stress, with tight connections to the ABA pathway.

5.1 Objective and approach

The primary goal of this thesis was to uncover new insights into ABA responses and explore the relationship between alternative splicing and the ABA pathway from a novel perspective. Previous studies have often investigated ABA responses using mutant lines from the same few laboratory strains or indirectly through studies of polymorphisms linked with ABA-mediated stress responses. Here, we used natural variation – defined as “the within-species phenotypic variation caused by spontaneously arising mutations that have been maintained in nature by any evolutionary process including, among others, artificial and natural selection” (Alonso-Blanco et al., 2009) – to identify Arabidopsis accessions with distinct ABA phenotypes.

The model plant *Arabidopsis thaliana* has been extensively used in the study of natural variation in stress responses due to its wide geographical distribution and its numerous natural variants. These variants are locally adapted and known to differ in their responses to stress at both the transcriptional and splicing levels. Thus, the aim of this study was to identify naturally occurring Arabidopsis accessions with distinct ABA phenotypes, as determined in Chapters 2 and 4, to shed new light into the regulation of ABA responses and the role of alternative splicing in ABA signalling.

5.2 Phenotypic screening and optimization

I began by screening natural variants to obtain Arabidopsis lines with different sensitivities to ABA, focusing on physiological responses controlled by the hormone. These responses are known to vary depending on the ABA concentration, the plant’s developmental stage, and the duration of stress. Furthermore, as a stress regulator, ABA triggers both rapid responses, such as stomatal closure, while the plant prepares for long-term responses, including biosynthesis of the hormone leading to a delay or arrest in plant growth (Kollist et al., 2019).

To identify candidate Arabidopsis accessions, we analysed both long-term responses, such as seedling development and root growth, and the rapid response of stomatal closure. Owing to the lack of easy, cost-effective protocols, we optimized a method to measure stomatal apertures (see Chapter 3). This method also allows the study of other morphological features, such as cellular patterns and stomatal density, while enabling long-term sample preservation. This optimized methodology allowed us to directly assess a process regulated by ABA: the closure of the stomatal pore.

5.3 Ecotype selection and transcriptomic analysis

From our initial collection of 24 natural variants, we narrowed our focus to two key accessions: Kn-0 and Col-0. Although other promising accessions like C24 exhibited notable ABA phenotypes, their inconsistent responses across different tissues or developmental stages made them less suitable for this study, albeit potentially interesting for future studies.

Importantly, Kn-0 was identified as the hyposensitive variant, consistently showing reduced ABA sensitivity compared to Col-0, which served as the hypersensitive control.

Although our two accessions come from nearby places – Kn-0 from Kaunas in Lithuania, and Col-0 presumably from Poland (though it was later transported to the United States of America, where it was named) – there is no correlation between the genetic and the geographical origin distances (Koornneef et al., 2004), nor between their endogenous ABA levels and climatic parameters (Kalladan et al.,2017).

We then conducted a comprehensive RNA-seq analysis to compare transcriptional and splicing changes between the two selected ecotypes, Col-0 and Kn-0. This high-resolution and in-depth analysis of the transcriptome allowed us to simultaneously assess gene expression and splicing variations. Previous research has shown that differences in the response to stress among Arabidopsis natural accessions may originate from sequence variation-induced alternative splicing (Wang et al., 2019b). Thus, considering the significant genetic differences between Kn-0 and Co-0 – such as the more than 600,000 SNPs and other sequence variants, including insertions and deletions identified by Gan et al. (2011) – we mapped reads to each genotype's genome to avoid potential mapping issues and loss of information.

5.4 Key findings and implications

Our results confirmed the role of ABA in regulating transcriptional responses and its impact on alternative splicing outcomes, further supporting the hypothesis that this post-transcriptional mechanism plays a critical role in plant stress responses. The Kn-0 ecotype exhibited a lower transcriptional response – fewer genes were altered, and the magnitude of change was lower – than Col-0, despite significant overlap in target genes and genes with similar functions being affected. In terms of numbers, the main differences were found within the ABA-downregulated genes, which were almost tripled in Col-0. These ABA-repressed genes were enriched in the catabolism of the ROS species hydrogen peroxide, a key signalling molecule that helps the plant spread stress signals (Huang et al., 2019a). Our results could indicate that, consistent with its increased sensitivity to ABA, the Col-0 ecotype is preventing, to a greater extent than Kn-0, the catabolism of this signalling molecule in order to transmit the stress signal systemically.

In all assessed phenotypic traits, Kn-0 consistently showed hyposensitivity relative to Col-0, suggesting that differences might lie within the core ABA signalling or homeostatic pathways. Kn-0 displayed lower overall expression and lower basal protein levels of SnRK2, a key component in ABA signalling, which may explain its reduced ABA sensitivity. Given that SnRK2s regulate multiple processes through phosphorylation during plant growth and responses to environmental stress, lower basal SnRK2 levels in Kn-0 could affect all aspects of the plant.

Furthermore, Kn-0 contained lower basal ABA levels, making it worthwhile to explore transcriptional differences between the two ecotypes in the absence of stress. In the ABA metabolic pathways, we found differences especially in BG1, a gene involved in reactivating inactive glycosylated ABA (ABA-GE). Measuring ABA-GE levels in both ecotypes would help confirm whether Kn-0 accumulates more ABA-GE. However, the high amounts of exogenous ABA used in our treatments may have masked the BG1-driven defects in the production of active ABA, with SnRK2 differences playing a more important role in the observed molecular and phenotypic variations.

Our study revealed that the Kn-0 accession, similar to its transcriptional response, exhibited fewer alternative splicing changes compared to Col-0. We found substantially fewer genes affected by splicing than by transcription, suggesting that either transcription is more sensitive to ABA-induced changes than splicing, or that our splicing detection threshold (10%, |IncDiff| > 0.1) was too high, potentially overlooking subtle yet important splicing changes. The functional categories of affected genes where similar between the ecotypes; however, the overlap of genes affected by splicing was limited. This suggests a specific role for splicing in the natural variation between Col-0 and Kn-0, contributing to their differential response to ABA. Although a minority, we identified several genes displaying shared ABA-induced alternative splicing events between the two ecotypes. Particularly those with ABA-related functions could serve as interesting targets for further studies.

Among the genes that exhibited ABA-induced splicing changes exclusively in Kn-0, two were particularly noteworthy: *ABA4*, which encodes a protein responsible for the conversion of violaxanthin into neoxanthin as part of the ABA biosynthesis pathway, and *PAC*, which is linked to the biosynthesis of carotenoids, the precursors of ABA. These alternative splicing events could lead to the production of truncated or altered proteins or target the proteins for degradation, potentially contributing to Kn-0's reduced ABA sensitivity. These findings highlight the importance of considering ecotype-specific responses.

Our results also indicate that relatively few genes are regulated at both the transcriptional and splicing levels, suggesting a complementary role for these two processes. This aligns with previous suggestions that alternative splicing operates independently from transcription (Ding et al., 2014; Calixto et al., 2018; Chen et al., 2018b; Martín et al., 2021). The recently released pangenome of *A. thaliana* reveals that core genes across 32 ecotypes are enriched in basic functions, such as transcriptional regulation and cellular homeostasis, while variable genes – those not present in all ecotypes – are involved in stress responses and secondary metabolic processes, suggesting a faster evolutionary rate for stress-related genes (Shen et al., 2023). This supports the notion that genes undergoing alternative splicing evolve more rapidly, likely aiding adaptation to adverse environmental conditions (Ganie and Reddy, 2021), and corroborates a crucial role for alternative splicing in stress responses.

5.5 Limitations of the study and future directions

The samples for our RNA-seq analysis represent only a snapshot of the full ABA response. A time-course analysis would provide more insights into the dynamic nature of ABA signalling, potentially identifying differences in the timing of responses between the ecotypes. We could also broaden the scope of our study by exploring alternative ABA biosynthetic pathways beyond ZEP, as well as considering ABA transport along the plant.

Additionally, we examined only two natural variants, which do not capture the full range of variation in *A. thaliana*'s response to ABA. To address this, future studies could incorporate a broader range of accessions and make use of a pan-genome or pan-transcriptome to capture all the genetic information, improving the detection of genetic variations and providing insights into the evolutionary dynamics of gene regulation at the transcription and splicing levels in the ABA response (Shen et al., 2023).

Future research should also explore basal transcriptomic differences between the ecotypes under non-stress conditions, beyond the few cases studied here, which might influence how each responds to the hormone.

While our results identify potential interesting targets underlying the differences in ABA sensitivity observed between Col-0 and Kn-0, these data will require further experimental validation to establish causality. For instance, gene variants from either ecotype containing the natural variants of the *SnRK2* promoter or SNPs in the *BG1* gene body, or transcript isoforms resulting from alternative splicing events specific to either ecotype could be introduced into the other ecotype to test whether they restore ABA sensitivity. However, one must take into account that the response to ABA involves multiple genes, and functionally testing them individually might not reveal the effects of each of our identified targets.

As ABA partially coordinates the response to osmotic stresses, such as drought, it would also be interesting to test the tolerance to these ABA-mediated stresses in the aforementioned complementation lines and measure the endogenous ABA levels under abiotic stress conditions.

For our splicing analysis, we used rMATS-turbo (Shen et al., 2014), focusing on identifying each specific alternative splicing event rather than the full transcript isoform. Understanding isoform usage frequency differences would be an interesting follow-up, as it would allow us to determine not only the overall gene expression levels but also which transcript isoforms are being expressed and produced. Combining the use of new technologies, such as ISO-seq, which allows for longer reads, with RNA-seq could provide a more comprehensive understanding of transcript isoform usage, potentially revealing new insights into ABA responses at both the transcriptional and splicing levels. Functional analyses should also be conducted to identify the alternative transcripts relevant to ABA responses.

Finally, understanding the interaction between splicing and transcription, potentially through chromatin alterations or the recruitment of splicing factors to the transcriptional machinery, could uncover players in the plant's responses to ABA.

5.6 Conclusion

The present study underscores the complexity of ABA-mediated stress responses and the role of alternative splicing in shaping these responses in different *Arabidopsis thaliana* natural accessions. While Col-0 and Kn-0 show clear differences in ABA sensitivity, the underlying mechanisms are multifaceted, involving both transcriptional and splicing changes. Future research should expand on these findings by incorporating more natural variants, conducting time-course analyses, and utilizing new sequencing technologies to help fully uncover the intricate network governing plant ABA responses.

5.7 References

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