Uniwersytet Śląski w Katowicach

Wydział Nauk Przyrodniczych

Instytut Biologii, Biotechnologii i Ochrony Środowiska

**EWA SYBILSKA**

# Molekularne mechanizmy sygnalizacji kwasu abscysynowego (ABA) podczas kiełkowania ziarniaków jęczmienia zwyczajnego (*Hordeum vulgare* L.) ze szczególnym uwzględnieniem roli jądrowego kompleksu wiążącego czapeczkę mRNA (CBC)

**PRACA DOKTORSKA**

**Praca doktorska wykonana pod kierunkiem:**

dr hab. Agata Daszkowska-Golec, prof. UŚ

Instytut Biologii, Biotechnologii i Ochrony Środowiska
Wydział Nauk Przyrodniczych
Uniwersytet Śląski w Katowicach

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**Podziękowania**

*Składam serdeczne podziękowania*

***Pani Promotor dr hab. Agacie Daszkowskiej-Golec, prof. UŚ***

*za merytoryczne wsparcie na każdym etapie mojej pracy naukowej, cenne wskazówki oraz przekazaną wiedzę. Dziękuję Pani za bycie prawdziwym wzorem naukowca z pasją oraz za pokazanie mi, czym jest dociekliwość naukowa, a także odwaga w stawianiu pytań, cierpliwość w poszukiwaniu odpowiedzi i radość z odkrywania tego, co wcześniej nieznane.*

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***Członkom Zespołu Genetyki i Genomiki Funkcjonalnej Roślin***

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**Spis treści**



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## 1. Wykaz prac naukowych wchodzących w skład cyklu stanowiącego podstawę rozprawy doktorskiej

**Publikacja 1 (P1): Sybilska E.**, Daszkowska-Golec A. (2023). Alternative splicing in ABA signaling during seed germination. *Frontiers in Plant Science*, 14, 1144990. https://doi.org/10.3389/fpls.2023.1144990

$IF_{2023}$: 4,1, MNiSW: 140 pkt

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**Publikacja 3 (P3): Sybilska E.**, Haddadi B.S., Mur L.A.J., Beckmann M., Hryhorowicz S., Suszyńska-Zajczyk J., Knaur M., Pławski A., Daszkowska-Golec A. (2025). Mapping the molecular signature of ABA-regulated gene expression in germinating barley embryos. *BMC Plant Biology*, 25(1), 619. https://doi.org/10.1186/s12870-025-06654-z

$IF_{2023}$: 4,3, MNiSW: 140 pkt

***Łączna suma IF = 12,2***

***Łączna suma punktów MNiSW = 420***



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## 3. Streszczenie

Kwas abscysynowy (ABA) pełni funkcję inhibitora kiełkowania nasion, jednak jego mechanizmy molekularne tego procesu, szczególnie u zbóż, są nadal nie w pełni poznane. Celem niniejszej rozprawy doktorskiej było zbadanie roli ABA w regulacji kiełkowania ziarniaków jęczmienia zwyczajnego (*Hordeum vulgare L.*), ze szczególnym uwzględnieniem jądrowego kompleksu wiążącego strukturę kapu mRNA (CBC; Cap-Binding Complex). Kompleks CBC jest heterodimerem złożonym z podjednostek CBP20 (Cap-Binding Protein 20) i CBP80. W badaniach wykorzystano ziarniaki i zarodki czterech genotypów jęczmienia: mutanta *hvcbp20.ab* oraz *hvcbp80.b,* podwójnego mutanta *hvcbp20.ab/hvcbp80.b*, a także odmianę rodzicielską 'Sebastian'. Zastosowano zintegrowane podejście badawcze obejmujące analizy fizjologiczne, transkryptomiczne oraz metabolomiczne. Wykazano, że mutacja obu genów kodujących podjednostki kompleksu CBC u mutanta *hvcbp20.ab/hvcbp80.b* znosi hamujący wpływ ABA na kiełkowanie, co sugeruje istnienie mechanizmu kompensacyjnego między *HvCBP20* a *HvCBP80*, gdyż pojedyncze mutanty wykazują wrażliwość na ABA. Fenotyp podwójnego mutanta *hvcbp20.ab/hvcbp80.b* powiązano ze znaczącymi zmianami w transkryptomie kiełkujących zarodków w odpowiedzi na ABA, obejmującymi zwiększoną liczbę genów i transkryptów o zróżnicowanej ekspresji, intensyfikację zdarzeń alternatywnego splicingu oraz interakcje pomiędzy regulacją sygnalizacji ABA, a sygnalizacją i metabolizmem brasinosteroidów. Zidentyfikowano również interakcje białkowe *in silico* pomiędzy podjednostkami kompleksu CBC a składnikami aparatu splicingowego. Ponadto w toku badań z wykorzystaniem sekwencjonowania w technologii długich odczytów PacBio opracowano referencyjny transkryptom jęczmienia genotypowo-specyficzny dla kiełkujących zarodków. W drugiej części pracy szczegółowo przeanalizowano działanie ABA w zarodkach odmiany 'Sebastian' na wczesnym etapie kiełkowania. Zintegrowano dane transkryptomiczne, metabolomiczne oraz przestrzenną transkryptomikę Visium. Ujawniono represyjne działanie ABA na ekspresję genów związanych z metabolizmem i wzrostem, przy jednoczesnej aktywacji procesów stresowych i szlaków fitohormonalnych. Wysoka korelacja między zmianami w transkryptomie i metabolomie wskazuje na skoordynowaną regulację ekspresji genów oraz procesów metabolicznych w kiełkujących zarodkach pod wpływem ABA. Ujawniono, że odpowiedź na ABA ma charakter tkankowo-specyficzny, z dominującą aktywnością transkrypcyjną w koleoptylu. Łącznie uzyskane wyniki wnoszą istotny wkład do badań nad molekularną i przestrzenną kontrolą procesu kiełkowania zbóż.



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## 4. Summary

Abscisic acid (ABA) acts as a seed germination inhibitor, but the molecular mechanism of this process, especially in crops, is still not fully understood. The aim of this doctoral dissertation was to investigate the role of ABA in the regulation of seed germination of barley (*Hordeum vulgare* L.), with particular emphasis on the nuclear mRNA Cap-Binding Complex (CBC), composed of the CBP20 (Cap-Binding Protein 20) and CBP80 subunits. We used grains and embryos of four barley genotypes: the double mutant *hvcbp20.ab/hvcbp80.b*, the single mutant *hvcbp20.ab* and *hvcbp80.b*, and their parent variety 'Sebastian'. An integrated research approach was applied, including physiological, transcriptomic and metabolomic analyses. Mutation of both CBC subunits was shown to abolish the inhibitory effect of ABA on germination of *hvcbp20.ab/hvcbp80.b*, suggesting a compensatory mechanism between *HvCBP20* and *HvCBP80*, as single mutants are sensitive to ABA. The double mutant *hvcbp20.ab/hvcbp80.b* phenotype was associated with significant changes in the transcriptome of germinating embryos in response to ABA, including increased numbers of differentially expressed genes and transcripts, enhanced alternative splicing, and interactions between ABA signaling and brassinosteroid signaling and metabolism. *In silico* protein-protein interactions between CBC subunits and components of the splicing machinery were also identified. In addition, a barley genotype-specific reference transcriptomic dataset for germinating embryos was elaborated. In the second part of the work, the effect of ABA on 'Sebastian' embryos at an early stage of germination was analyzed in detail. Transcriptomic, metabolomic and Visium spatial transcriptomic data were combined. ABA revealed a repressive effect on the expression of genes related to metabolism and growth, while simultaneously activating stress processes and phytohormonal pathways. A high correlation between the transcriptome and the metabolome changes indicates a coordinated regulation of gene expression and metabolic processes in germinating embryos after ABA treatment. It was revealed that the response to ABA is tissue-specific, with dominant transcriptional activity in the coleoptile. Together, the obtained results make a significant contribution to the research on the molecular and spatial control of the crop germination process.



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## 5. Wykaz stosowanych skrótów

ABA (kwas abscysynowy; abscisic acid)

AS (alternatywny splicing; alternative splicing)

AUX (auksyna; auxin)

BR (brasinosteroidy; brassinosteroids)

cDNA (DNA komplementarne; complementary DNA)

DAI (dzień po imbibicji; day after imbibition)

DAS (różnicowe alternatywne składanie genów; differential alternative splicing)

DEG (geny o zróżnicowanej ekspresji; differentially expressed genes)

DET (transkrypty o zróżnicowanej ekspresji; differentially expressed transcripts)

DTU (różnicowo wykorzystywane transkrypty; differential transcript usage)

GA (gibereliny; gibberellins)

GO (ontologia genów; gene ontology)

IAA (kwas indolilo-3-octowy; indole-3-acetic acid)

JA (kwas jasmonowy; jasmonic acid)

KEGG (Baza Genów i Genomów Kyoto; Kyoto Encyclopedia of Genes and Genomes)

LC-MS/MS (chromatografia cieczowa sprzężona ze spektrometrią mas; liquid chromatography–tandem mass spectrometry)

NMD (mechanizm degradacji mRNA zawierającego przedwczesny kodon stop; nonsense-mediated decay)

PTC (przedwczesny kodon stop; premature termination codon)

RNA-seq (sekwencjonowanie RNA; RNA sequencing)

TF (czynnik transkrypcyjny; transcription factor)

WT (typ dziki; wild-type)

*Skróty nazw genów i białek zostały rozwinięte przy ich pierwszym użyciu w tekście autoreferatu, zgodnie z powszechnie akceptowanymi zasadami nomenklatury genetycznej stosowanej w biologii molekularnej roślin.*



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## 6. Autoreferat

### 6.1. Wprowadzenie

#### 6.1.1. Kwas abscysynowy (ABA) jako kluczowy regulator procesu kiełkowania nasion

Kiełkowanie nasion, będące pierwszym etapem cyklu życiowego roślin, jest fundamentalnym procesem, który warunkuje ich prawidłowy wzrost i rozwój. Obejmuje on szereg przemian biochemicznych i fizjologicznych, które rozpoczynają się od pęcznienia nasion (imbibicji) i prowadzą do inicjacji wzrostu korzenia zarodkowego (Bradford i Nonogaki, 2007). Zahamowanie kiełkowania w niesprzyjających warunkach środowiskowych stanowi istotny mechanizm adaptacyjny, który pozwala roślinom przetrwać niekorzystny okres i rozpocząć wzrost dopiero wtedy, gdy warunki staną się optymalne. Kiełkowanie kontrolowane jest przez liczne czynniki środowiskowe, takie jak temperatura, dostęp do wody, światło oraz obecność tlenu. Na poziomie molekularnym proces ten zależy od złożonych interakcji fitohormonalnych i genetycznych. Kluczową rolę odgrywa równowaga między kwasem abscysynowym (ABA), który hamuje kiełkowanie, a gibereliną (GA), która je stymuluje (Finch-Savage i Leubner-Metzger, 2006; Liu i Hou, 2018; Vishal i Kumar, 2018; Carrera-Castaño *i in.*, 2020; Sano i Marion-Poll, 2021; Nautiyal *i in.*, 2023). ABA jest głównym fitohormonem stresowym, który reguluje rozwój roślin oraz pełni fundamentalną rolę w ich adaptacji do różnorodnych czynników abiotycznych. Działanie ABA jako inhibitora kiełkowania opiera się na hamowaniu ekspresji genów związanych z kiełkowaniem oraz aktywacji szlaków molekularnych, które utrzymują stan uśpienia (Kermode, 2005; Finkelstein *i in.*, 2008; Nonogaki, 2019; Ali *i in.*, 2022). Poziom endogennego ABA w komórce jest rezultatem aktywnej biosyntezy ABA, której kluczowym etapem jest reakcja katalizowana przez enzym 9-cis-epoksykarotenoidową dioksygenazę (NCED; 9-cis epoxycarotenoid dioxygenase). *NCED6* i *NCED9* wykazują wysoką ekspresję odpowiednio w bielmie i zarodku, kontrolując poziom ABA podczas rozwoju i kiełkowania nasion (Lefebvre *i in.*, 2006; Martínez-Andújar *i in.*, 2011). Mutacje w tych genach prowadzą do znacznego obniżenia poziomu ABA w nasionach, skutkując ich przedwczesnym kiełkowaniem (Lefebvre *i in.*, 2006). Oprócz biosyntezy ABA, istotnym mechanizmem kontroli poziomu endogennego ABA jest jego katabolizm, który zachodzi głównie z udziałem enzymów z rodziny CYP707A (CYTOCHROME P450 707A), odpowiedzialnych za 8′-hydroksylację i degradację ABA (Nambara i Marion-Poll, 2005). Wraz z poprawą warunków środowiskowych, degradacja ABA oraz jednoczesna



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biosynteza GA, prowadzi do aktywacji zarodka, co umożliwia przerwanie stanu spoczynku, rozerwanie osłonki nasiennej i rozpoczęcie procesu kiełkowania (Farooq *i in.*, 2022). Taka dynamiczna regulacja ekspresji genów zaangażowanych zarówno w biosyntezę, jak i katabolizm ABA, umożliwia precyzyjne dostosowanie poziomu ABA do zmieniających się warunków środowiskowych. Dla skutecznej reakcji roślin na ABA kluczowe znaczenie ma nie tylko jego akumulacja, lecz także zdolność komórek roślinnych do percepcji i transdukcji sygnału fitohormonalnego, która determinuje efektywność odpowiedzi fizjologicznej na poziomie molekularnym. W odpowiedzi na warunki stresowe, aktywowany zostaje szlak sygnałowy ABA, który obejmuje receptory PYR/PYL (PYRABACTIN RESISTANCE/PYR-LIKE PROTEIN), fosfatazy PP2C (PROTEIN PHOSPHATASE 2C), kinazy SnRK2 (SNF1-RELATED PROTEIN KINASE 2), kontrolujące ekspresję genów zależnych od ABA. Fosfatazy PP2C pełnią rolę negatywnych regulatorów tego szlaku, hamując aktywność kinaz SnRK2 poprzez ich defosforylację. W obecności ABA dochodzi do zahamowania PP2C, co umożliwia aktywację SnRK2, a w konsekwencji fosforylację czynników transkrypcyjnych i indukcję ekspresji genów odpowiedzialnych za utrzymanie stanu spoczynku nasion (Fujii *i in.*, 2009; Park *i in.*, 2009; Umezawa *i in.*, 2009). Głównymi efektorami odpowiedzi na sygnał ABA w nasionach są czynniki transkrypcyjne z rodziny bZIP, w tym ABI5 (ABA INSENSITIVE 5), który wiążąc się z motywem ABRE (ABA-RESPONSIVE ELEMENT) w promotorach genów odpowiedzi na ABA, hamuje kiełkowanie nasion (Lopez-Molina i Chua, 2000; Skubacz *i in.*, 2016). Co więcej, poprzez regulację ekspresji receptorów PYR/PYL, ABI5 inicjuje sprzężenie zwrotne utrzymujące aktywność szlaku sygnalizacji ABA w nasionach (Zhao *i in.*, 2020). Mutacje w genie *ABI5* prowadzą do niewrażliwości na ABA podczas kiełkowania, co wskazuje na jego rolę jako pozytywny regulator odpowiedzi na ABA (Finkelstein, 1994; Finkelstein i Lynch, 2000; Lopez-Molina i Chua, 2000). Z kolei ABI3 to czynnik transkrypcyjny z domeną B3, który należy do grupy regulatorów LAFL (LEC1 (LEAFY COTYLEDON 1), ABI3, FUS3 (FUSCA 3), LEC2), kontrolujących rozwój zarodka, dojrzewanie i kiełkowanie nasion oraz przejście do wzrostu wegetatywnego (Parcy *i in.*, 1997; Chen i Du, 2022). ABI3 współdziała z ABI5, wzmacniając jego aktywność i nasilając odpowiedź na ABA (Nakamura *i in.*, 2001). Wykazano również, że ABI3 działa powyżej ABI5 w szlaku sygnałowym ABA (Lopez-Molina *i in.*, 2002).



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Chociaż ABA odgrywa kluczową rolę w hamowaniu kiełkowania, efekt ten wynika z działania złożonej sieci sygnałowej, w której uczestniczą również inne fitohormony. Dane literaturowe wskazują, że gibereliny, etylen, brasinosteroidy oraz cytokininy osłabiają działanie ABA, podczas gdy jasmoniany i auksyny zazwyczaj je wzmacniają (Xie *i in.*, 2007; Daszkowska-Golec, 2011; Wang *i in.*, 2011; Harrison, 2012; Linkies i Leubner-Metzger, 2012; Guan *i in.*, 2014; Hu i Yu, 2014; Shu *i in.*, 2016; Tuan *i in.*, 2018; Varshney i Majee, 2021; Parwez *i in.*, 2022; Mei *i in.*, 2023; Sybilska i Daszkowska-Golec, 2023). Warto jednak podkreślić, że ich wpływ na regulację kiełkowania nie jest jednoznaczny i może różnić się w zależności od gatunku, stadium rozwoju nasion oraz warunków środowiskowych. Wieloaspektowość działania ABA a także złożoność jego funkcjonowania i interakcji z innymi fitohormonami, podkreślają konieczność dalszych badań, szczególnie w kontekście jego roli jako centralnego regulatora w sieci sygnałowej kontrolującej kiełkowanie nasion.

### 6.1.2. Rola kompleksu CBC (Cap-Binding Complex) jako modulatora odpowiedzi ABA podczas kiełkowania nasion

Kompleks CBC (Cap-Binding Complex) jest białkowym heterodimerem, składającym się z dwóch podjednostek: mniejszej CBP20 (Cap-Binding Protein 20) oraz większej CBP80. CBC wiąże się ze specyficzną strukturą nukleotydową znajdującą się na końcu 5' nowo syntetyzowanego transkryptu – kapem mRNA (czapeczką mRNA; $m^7G$, 7-metyloguanozyną). Struktura kapu pełni rolę ochronną, zapobiegając degradacji mRNA, oraz jest kluczowa dla jego dalszego przetwarzania. Podjednostka CBP20 bezpośrednio wiąże czapeczkę mRNA, podczas gdy CBP80 stabilizuje CBP20 i pełni funkcję regulatorową. Kompleks CBC uczestniczy w wielu fundamentalnych procesach związanych z metabolizmem RNA w komórce, takich jak transkrypcja, splicing konstytutywny i alternatywny, poliadenylacja, eksport mRNA z jądra do cytoplazmy oraz biogeneza mikroRNA. Kompleks CBC występuje również u innych eukariontów, w tym u zwierząt, ludzi oraz u drożdży. Jego sekwencje nukleotydowe i białkowe są wysoce konserwowane ewolucyjnie, co świadczy o fundamentalnym znaczeniu kompleksu dla prawidłowego funkcjonowania komórki (Kmieciak *i in.*, 2002; Kierzkowski *i in.*, 2009; Gonatopoulos-Pournatzis i Cowling, 2014; Daszkowska-Golec *i in.*, 2017; Daszkowska-Golec, 2018). CBP20 i CBP80, zostały zidentyfikowane jako negatywne regulatory szlaku sygnalizacyjnego ABA. Potwierdzono to zarówno u modelowej rośliny *Arabidopsis thaliana*, jak i u jęczmienia, gdzie wykazano,



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że mutanty w tych genach wykazują zwiększoną wrażliwość na ABA podczas kiełkowania nasion (Hugouvieux *i in.*, 2001; Papp *i in.*, 2004; Jäger *i in.*, 2011; Daszkowska-Golec *i in.*, 2013, 2017). Co więcej, mutacje w *CBP20* i *CBP80* nadają roślinom tolerancję na niedobór wody, co objawia się m.in. szybszym zamykaniem aparatów szparkowych, wyższą zawartością wody w tkankach oraz zmianami anatomicznymi liści, takimi jak zwiększona depozycja wosków epikutikularnych, zwiększona liczba trichomów i zmodyfikowane rozmieszczenie aparatów szparkowych. Dodatkowo, mutanty te wykazują zmienioną ekspresję genów związanych z metabolizmem i sygnalizacją ABA, co potwierdza ich aktywny udział w regulacji odpowiedzi na stres zależnej od ABA (Hugouvieux *i in.*, 2001, 2002; Papp *i in.*, 2004; Jäger *i in.*, 2011; Pieczynski *i in.*, 2013; Daszkowska-Golec *i in.*, 2017, 2020). Wiedza na temat roli kompleksu CBC w sygnalizacji ABA jest wciąż ograniczona, szczególnie w przypadku jęczmienia, który jest istotną rośliną agronomiczną. Dotychczasowe badania koncentrowały się głównie na pojedynczych mutantach w genach *CBP20* i *CBP80* u *Arabidopsis thaliana* oraz jęczmienia, a także na podwójnym mutancie Arabidopsis. W związku z tym podkreśla się konieczność przeprowadzenia dalszych badań nad efektem podwójnej mutacji w tych genach u jęczmienia, co pozwoli na dokładniejsze zrozumienie roli kompleksu CBC w regulacji odpowiedzi na ABA, zwłaszcza w kontekście kiełkowania ziarniaków, ale także adaptacji roślin do stresów abiotycznych, takich jak susza. Badania te mają kluczowe znaczenie, szczególnie w obliczu postępujących zmian klimatycznych, które stanowią jedno z największych wyzwań współczesnego rolnictwa.

### 6.1.3. Alternatywny splicing (AS) i rola kompleksu CBC (Cap-Binding Complex) w jego regulacji podczas kiełkowania nasion

Alternatywny splicing (AS) to jeden z etapów dojrzewania pre-mRNA (pre-messenger RNA) zachodzący w jądrze komórkowym ko- i post-transkrypcyjnie, polegający na wycinaniu intronów oraz selektywnym usuwaniu lub zachowywaniu określonych eksonów, które mogą być łączone w różnych konfiguracjach. Dzięki temu jedna sekwencja genomowa może determinować wiele wariantów (izoform) transkryptów mRNA, co prowadzi do syntezy białek o zróżnicowanej strukturze i funkcji, co znacząco zwiększa różnorodność proteomu (Nilsen i Graveley, 2010; Verta i Jacobs, 2022) (Ryc. 1).



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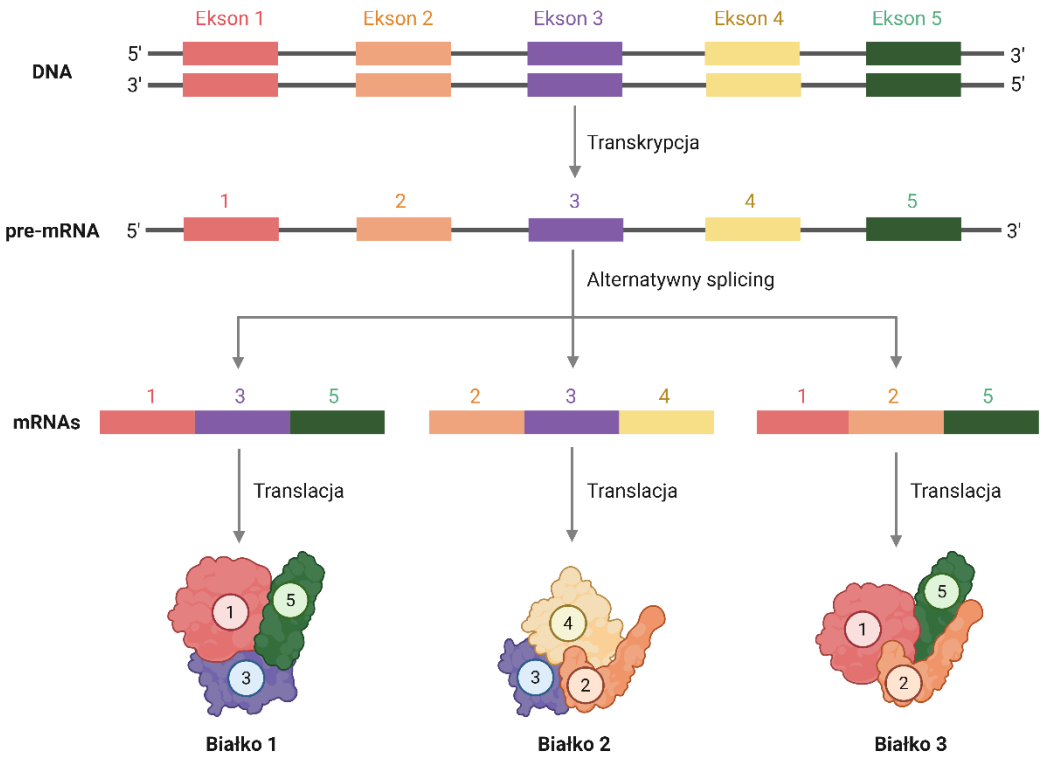


Rycina 1. Schematyczne przedstawienie procesu alternatywnego splicingu jako mechanizmu prowadzącego do powstawania różnych izoform białkowych. Rycina przygotowana z wykorzystaniem BioRender (https://biorender.com/).

Podczas splicingu konstytutywnego wszystkie eksony zostają zachowane, a introny usunięte z pierwotnego transkryptu pre-mRNA, co prowadzi do powstania jednej formy dojrzałego mRNA. Natomiast w przypadku AS wyróżnia się kilka typów zdarzeń prowadzących do powstawania różnych izoform mRNA takich jak:

- wzajemnie wykluczające się eksony (mutually exclusive exons), w których spośród dwóch alternatywnych eksonów w dojrzałym mRNA zachowany zostaje wyłącznie jeden ekson;
- pominięcie eksonu (exon skipping) polegające na usunięciu danego eksonu z pierwotnego transkryptu pre-mRNA;
- alternatywne miejsce donorowe (alternative 5' splice site) i alternatywne miejsce akceptorowe (alternative 3' splice site), w których dochodzi odpowiednio do przesunięcia 5′ lub 3′ miejsca splicingu w pre-mRNA;
- retencja intronu (intron retention), w której dany intron zostaje zachowany w dojrzałym mRNA (Laloum *i in.*, 2018; Chaudhary *i in.*, 2019).



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Powstałe w wyniku alternatywnego splicingu warianty transkryptów mRNA mogą kodować białka pełnej długości, skrócone lub całkowicie niefunkcjonalne. W niektórych przypadkach zdarzenia splicingowe prowadzą do zmiany ramki odczytu (frameshift) i powstania przedwczesnych kodonów stop (PTC; premature termination codon), co może skutkować degradacją transkryptu poprzez mechanizm NMD (nonsense-mediated decay). Powstające białka mogą również różnić się stabilnością, lokalizacją komórkową i aktywnością biologiczną (Syed *i in.*, 2012; Filichkin *i in.*, 2015). Ciekawym przykładem zmian splicingowych są zdarzenia typu NAGNAG, polegające na alternatywnym wykorzystaniu dwóch blisko położonych miejsc akceptorowych, co skutkuje insercją lub delecją trzech nukleotydów w mRNA. W efekcie powstaje białko różniące się tylko jednym aminokwasem, co może mieć różny efekt w zależności od lokalizacji tej zmiany w strukturze białka (Busch i Hertel, 2012).

Splicing pre-mRNA przeprowadzany jest przez spliceosom, będącym dużym kompleksem rybonukleoprotein, składającym się z małych jądrowych RNA (snRNA; small nuclear RNA) oraz licznych białek (Reddy, 2007; Gehring i Roignant, 2021). Kluczowe podjednostki spliceosomu, takie jak U1, U2, U4/U6 oraz U5, precyzyjnie rozpoznają sekwencje graniczne intronów i eksonów, zapewniając prawidłowe przetwarzanie transkryptów. Proces ten przebiega w kilku etapach, podczas których dochodzi do stopniowego montażu, aktywacji i katalizy w spliceosomie. W pierwszej fazie U1 przyłącza się do miejsca donorowego intronu (5'ss; 5' splice sites), a U2 do sekwencji rozgałęzienia (BPS; branch point sequence). Następnie do kompleksu dołączają U4/U6 i U5, tworząc w pełni funkcjonalny spliceosom. Po serii zmian konformacyjnych i dwóch etapach katalitycznych następuje wycięcie intronu oraz połączenie eksonów, prowadząc do powstania dojrzałego mRNA (Reddy, 2007; Köster *i in.*, 2014; Gehring i Roignant, 2021). Szczegółowy mechanizm działania spliceosomu oraz znaczenie AS w regulacji ekspresji genów u roślin zostały opisane w pracy przeglądowej (Sybilska i Daszkowska-Golec, 2023) **(P1)**.

Kompleks CBC (Cap-Binding Complex) pełni istotną funkcję w regulacji alternatywnego splicingu. Wiadomo, że CBC poprzez interakcję z białkiem SE (SERRATE) oraz podjednostką U1 spliceosomu, wpływa na precyzyjną selekcję miejsc splicingowych w pre-mRNA, a także odpowiada za rekrutację czynników splicingowych (Laubinger *i in.*, 2008; Raczynska *i in.*, 2010; Gonatopoulos-Pournatzis i Cowling, 2014; Daszkowska-Golec, 2018). Badania nad mutantami *cbp20* i *cbp80* oraz podwójnym mutantem *cbp20/80* u *Arabidopsis thaliana* wykazały, że brak funkcjonalnego CBC



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prowadzi do istotnych zaburzeń w AS pre-mRNA, takich jak zwiększona retencja intronów oraz zmieniona ekspresja alternatywnych izoform mRNA. Zasugerowano, że szczególnie istotną rolę w tym procesie odgrywa większa podjednostka kompleksu - CBP80 (Raczynska *i in.*, 2010). Coraz więcej badań wskazuje na kluczową rolę AS w regulacji odpowiedzi roślin na czynniki środowiskowe. Co ciekawe, ujawniono że przedtraktowanie ABA wspomaga odporność roślin na suszę poprzez mechanizm alternatywnego splicingu (Collin *i in.*, 2025). Mimo że AS stanowi istotny mechanizm regulacyjny integrujący sygnalizację ABA z kontrolą procesu kiełkowania, wciąż brakuje szczegółowych badań dotyczących jego znaczenia podczas ABA-zależnego kiełkowania nasion. W pracy przeglądowej **(P1)** Sybilska i Daszkowska-Golec (2023), stanowiącej syntetyczne podsumowanie aktualnego stanu wiedzy na temat roli procesu alternatywnego splicingu w procesie kiełkowania nasion, omówiono znane warianty splicingowe genów zaangażowanych w proces kiełkowania nasion i przedstawiono ich potencjalny wpływ na funkcjonalność powstających białek. Opisano izoformy *HAB1* (*HYPERSENSITIVE TO ABA1*), *ABI3*, *ABI5*, *PIF6* (*PHYTOCHROME-INTERACTING FACTOR 6*), *CBP80* (*ABH1; ABA HYPERSENSITIVE 1*) i *DOG1* (*DELAY OF GERMINATION 1*), które uczestniczą w transdukcji sygnału ABA lub pośrednio regulują odpowiedź nasion na ten fitohormon (McKibbin *i in.*, 2002; Wilkinson *i in.*, 2005; Fan *i in.*, 2007; Zou *i in.*, 2007; Gagete *i in.*, 2009; Penfield *i in.*, 2010; Sugliani *i in.*, 2010; Gao *i in.*, 2013; Wang *i in.*, 2015, 2018; Née *i in.*, 2017; Punzo *i in.*, 2020*b*; Bi *i in.*, 2021; Lalanne *i in.*, 2021; Zhang *i in.*, 2022). Podkreślono również, że AS modyfikuje także transkrypty genów kodujących czynniki splicingowe, takie jak PTB1 (POLYPYRIMIDINE TRACT BINDING PROTEIN 1), PTB2, SR45 (SERINE/ARGININE-RICH 45) i SR45a, których różne izoformy mogą w odmienny sposób wpływać na ekspresję, swój własny splicing oraz AS innych transkryptów, modulując tym samym odpowiedź nasion na ABA i dynamikę procesu kiełkowania (Carvalho *i in.*, 2010; Stauffer *i in.*, 2010; Rühl *i in.*, 2012; Narsai *i in.*, 2017; Albaqami *i in.*, 2019; Li *i in.*, 2021).



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## 7. Cel i założenia rozprawy doktorskiej

Celem niniejszej rozprawy doktorskiej było zbadanie molekularnych mechanizmów sygnalizacji kwasu abscysynowego (ABA) podczas kiełkowania ziarniaków jęczmienia zwyczajnego (*Hordeum vulgare* L.) ze szczególnym uwzględnieniem roli jądrowego kompleksu wiążącego strukturę kapu mRNA (czapeczkę mRNA) (CBC; Cap-Binding Complex). Kwas abscysynowy to fitohormon, który odgrywa istotną rolę w hamowaniu kiełkowania nasion, szczególnie w odpowiedzi na niekorzystne warunki środowiskowe. Chociaż funkcja ABA jako inhibitora tego procesu została dobrze udokumentowana, nadal brakuje szczegółowej wiedzy na temat molekularnych mechanizmów jego działania oraz szlaków regulatorowych aktywowanych w kiełkujących nasionach w odpowiedzi na ABA. Z uwagi na znaczenie jęczmienia jako rośliny o istotnym potencjale agronomicznym, ograniczony stan wiedzy na temat tej regulacji uzasadnia konieczność prowadzenia badań nad molekularnym działaniem ABA u tego gatunku. W niniejszej rozprawie doktorskiej skupiono się na analizie roli jednego z kluczowych regulatorów tego szlaku, jakim jest jęczmienny kompleks CBC, na etapie kiełkowania ziarniaków w obecności ABA. Kompleks CBC, złożony z dwóch podjednostek, mniejszej, kodowanej przez gen *HvCBP20* (*Cap-Binding Protein 20*), oraz większej, kodowanej przez *HvCBP80*, zaangażowany jest w procesy związane z metabolizmem RNA, uczestnicząc m.in. w dojrzewaniu i transporcie mRNA, a także w regulacji procesu alternatywnego splicingu (AS). Ponadto, w niniejszej pracy doktorskiej przeprowadzono analizę wpływu tego fitohormonu na kiełkujące ziarniaki jęczmienia odmiany ‘Sebastian’ w precyzyjnie określonym interwale czasowym, obejmującym pierwszy dzień po zakończeniu stratyfikacji, aby uchwycić najwcześniejsze etapy molekularnej odpowiedzi na sygnał ABA.

Na podstawie dotychczasowej wiedzy literaturowej oraz wcześniejszych badań, postawiono następujące **<u>hipotezy badawcze</u>**:

**H1:** Kompleks CBC uczestniczy w regulacji alternatywnego splicingu w odpowiedzi na ABA, potencjalnie modulując ekspresję genów kontrolujących kiełkowanie ziarniaków
i adaptację do warunków stresowych **(P2)**.

**H2:** Kompleks CBC reguluje wzajemne oddziaływania między ABA a innymi fitohormonami kontrolując zdolność do kiełkowania ziarniaków jęczmienia **(P2)**.



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**H3:** ABA indukuje specyficzne zmiany w ekspresji genów i metabolomie w kiełkujących zarodkach jęczmienia, pełniąc funkcję sygnału molekularnego, który koordynuje reakcje adaptacyjne i interakcje fitohormonalne w odpowiedzi na stres **(P3)**.
**H4:** Ekspresja genów zależnych od ABA wykazuje tkankową specyficzność w różnych obszarach kiełkujących zarodków jęczmienia **(P3)**.

Aby zweryfikować postawione hipotezy badawcze możliwie w najszerszym ujęciu, badania przeprowadzono wykorzystaniem zróżnicowanego materiału genetycznego jęczmienia na różnych poziomach biologicznej regulacji.
1. Weryfikacja hipotez **(H1-H2)**, dotyczących roli kompleksu CBC w ABA-zależnym procesie kiełkowania, została przeprowadzona z wykorzystaniem ziarniaków i kiełkujących zarodków pojedynczych mutantów w genie *HvCBP20* (*hvcbp20.ab*) oraz *HvCBP80* (*hvcbp80.b*) oraz podwójnego mutanta *hvcbp20.ab/hvcbp80.b*, niosącego mutacje w obu genach kodujących podjednostki kompleksu CBC, a także odmiany rodzicielskiej 'Sebastian' **(P2)**. Analizy prowadzono na trzech poziomach biologicznej regulacji:

- **Poziom fizjologiczny** – analiza fenotypu podwójnego mutanta *hvcbp20.ab/hvcbp80.b* oraz pojedynczych mutantów *hvcbp20.ab*, *hvcbp80.b* i odmiany rodzicielskiej 'Sebastian' w odpowiedzi na ABA podczas kiełkowania ziarniaków w celu określenia ich wrażliwości na ten fitohormon.
- **Poziom transkryptomiczny** – analiza ekspresji genów i transkryptów w kiełkujących zarodkach czterech badanych genotypów z wykorzystaniem sekwencjonowania transkryptomu (RNA-seq) metodą krótkich odczytów Illumina oraz długich odczytów PacBio w celu określenia globalnych zmian w transkryptomie pod wpływem ABA oraz charakterystyki procesu alternatywnego splicingu, a także do opracowania referencyjnego genotypowo-specyficznego transkryptomu jęczmienia dla kiełkujących zarodków.
- **Poziom metabolomiczny** – oznaczenie poziomu metabolitów roślinnych w kiełkujących zarodkach czterech badanych genotypów w warunkach kontrolnych oraz w obecności ABA z wykorzystaniem chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (LC-MS/MS) w celu zbadania wpływu CBC na regulację poziomu fitohormonów podczas procesu kiełkowania.



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2. Weryfikacja hipotez **(H3-H4)** dotyczących wpływu egzogennego ABA na kiełkujące zarodki została przeprowadzona z wykorzystaniem odmiany ‘Sebastian’ **(P3)** na dwóch poziomach biologicznej regulacji:

- **Poziom transkryptomiczny** – (i) globalna analiza ekspresji genów przy użyciu technologii sekwencjonowania transkryptomu metodą Illumina oraz analiza wzbogacenia ścieżek biologicznych w celu zbadania molekularnych mechanizmów regulacyjnych związanych z działaniem ABA w kiełkujących zarodkach jęczmienia odmiany ‘Sebastian’. Ponadto przeprowadzono analizę porównawczą transkryptomu kiełkujących zarodków w obecności ABA z dostępnymi publicznie danymi transkryptomicznymi różnych tkanek w obrębie rozwijających się ziarniaków w celu identyfikacji specyficznych procesów biologicznych związanych z odpowiedzią na egzogenny ABA; (ii) przestrzenna analiza lokalizacji ekspresji genów regulowanych przez ABA w różnych tkankach zarodka jęczmienia odmiany ‘Sebastian’ w celu ustalenia ich tkankowej specyficzności podczas kiełkowania, przy użyciu metody Visium Spatial Transcriptomics (10x Genomics).
- **Poziom metabolomiczny** – analiza metabolomu z wykorzystaniem chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (LC-MS/MS) w zarodkach jęczmienia odmiany ‘Sebastian’, pod wpływem ABA w celu określenia zmian w metabolomie oraz identyfikacji fitohormonalnych interakcji związanych z procesem kiełkowania.

Zastosowane podejście badawcze, łączące analizy fizjologiczne z wielopoziomową analizą molekularną, umożliwiło szczegółową charakterystykę roli kompleksu CBC w regulacji kiełkowania ziarniaków jęczmienia w odpowiedzi na ABA oraz dogłębne zbadanie mechanizmów działania ABA we wczesnym, ściśle określonym interwale czasowym podczas procesu kiełkowania.



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## 8. Materiał badawczy, warunki eksperymentalne i metody

### 8.1.1 Materiał badawczy

Materiał badawczy wykorzystany w niniejszej rozprawie doktorskiej stanowiły ziarniaki oraz kiełkujące zarodki:

- jęczmienia zwyczajnego (*Hordeum vulgare* L.) odmiany 'Sebastian' **(P2, P3)**

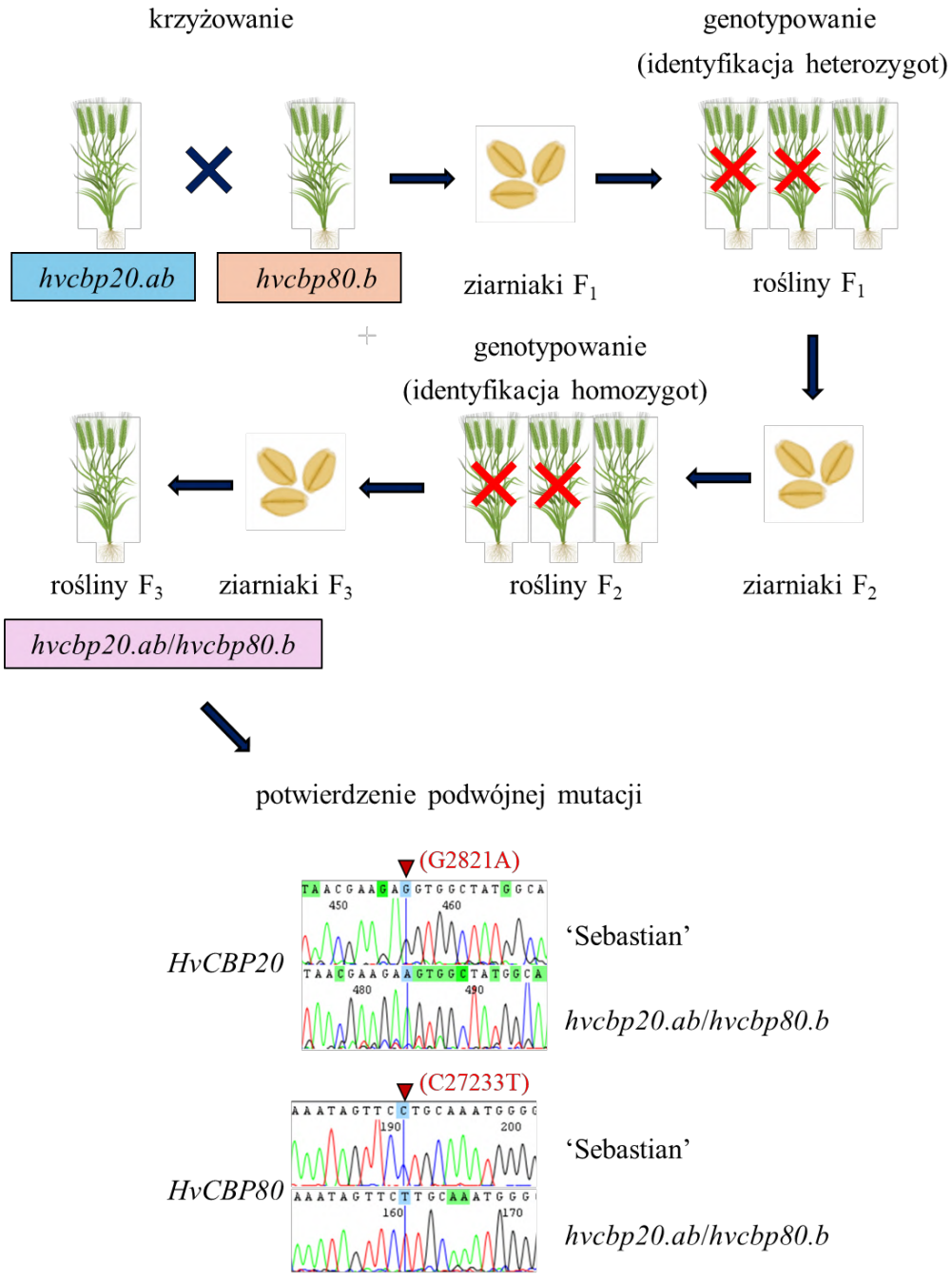
a także trzy linie mutantów TILLING, wyprowadzone w ramach wcześniej prowadzonych prac badawczych w Zespole Genetyki i Genomiki Funkcjonalnej Roślin w tle genetycznym odmiany 'Sebastian', stanowiącej odmianę rodzicielską dla populacji *Hor*TILLUS (*Hordeum vulgare* – TILLING – University of Silesia) (Szarejko *i in.*, 2017; Szurman-Zubrzycka *i in.*, 2018) **(P2)**:

- mutant *hvcbp20.ab*, niosący mutację punktową (G2821A) w genie *HvCBP20* (*CAP-BINDING PROTEIN 20*; BaRT2v18chr6HG306340) (Daszkowska-Golec *i in.*, 2017),
- mutant *hvcbp80.b*, niosący mutację punktową (C27233T) w genie *HvCBP80* (*CAP-BINDING PROTEIN 80*; BaRT2v18chr4HG195950),
- podwójny mutant *hvcbp20.ab/hvcbp80.b*, niosący mutacje punktowe w obu genach: *HvCBP20* (G2821A) oraz *HvCBP80* (C27233T).

Pojedyncze mutanty zostały zidentyfikowane w populacji *Hor*TILLUS, wyprowadzonej w Zespole Genetyki i Genomiki Funkcjonalnej Roślin w Uniwersytecie Śląskim w Katowicach poprzez traktowanie ziarniaków jęczmienia odmiany 'Sebastian' mutagenami: azydkiem sodu ($NaN_3$) oraz N-metylonitrozomocznikiem (MNU) (Szarejko *i in.*, 2017; Szurman-Zubrzycka *i in.*, 2018). Pojedyncze mutanty wyselekcjonowano metodą TILLING w pokoleniu $M_2$, a następnie skrzyżowano wstecznie z roślinami typu dzikiego, w celu eliminacji niepożądanych zmian genetycznych. Natomiast podwójnego mutanta *hvcbp20.ab/hvcbp80.b* uzyskano poprzez genotypowanie potomstwa $F_2$ po skrzyżowaniu pojedynczych mutantów *hvcbp20.ab* i *hvcbp80.b*. W niniejszej pracy obecność mutacji w genach *HvCBP20* i *HvCBP80* u roślin pokolenia $F_3$ potwierdzono metodą sekwencjonowania Sangera, przeprowadzoną w trzech powtórzeniach biologicznych dla każdego z badanych osobników (Ryc. 2).



19:6322377762




Rycina 2. Schemat ilustrujący wyprowadzenie podwójnego mutanta *hvcbp20.ab*/*hvcbp80.b*. Na podstawie: Sybilska i *in*., 2024 **(P2)**, zmodyfikowano.



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### 8.1.2 Warunki eksperymentalne

Ziarniaki jęczmienia sterylizowano w 20% roztworze podchlorynu sodu przez 20 minut a następnie 3 razy płukano w sterylnej wodzie przez 5 minut. Następnie umieszczono je na szalkach Petriego (90 mm) z trzema warstwami filtrów papierowych Whatman i 5 ml sterylnej wody w przypadku kontroli lub 5 ml roztworu 75 µM ABA (cis–trans-ABA; Sigma-Aldrich, kat. nr 862169). Przez 4 dni ziarniaki poddano stratyfikacji w temperaturze 4°C w ciemności. Po zakończeniu stratyfikacji, szalki z ziarniakami przenoszono do pokoju hodowlanego o kontrolowanych warunkach: temperatura 22°C, fotoperiod 16 godzin światła / 8 godzin ciemności, natężenie światła 200 µmol $m^{-2}$ $s^{-1}$. W opisanych powyżej warunkach:

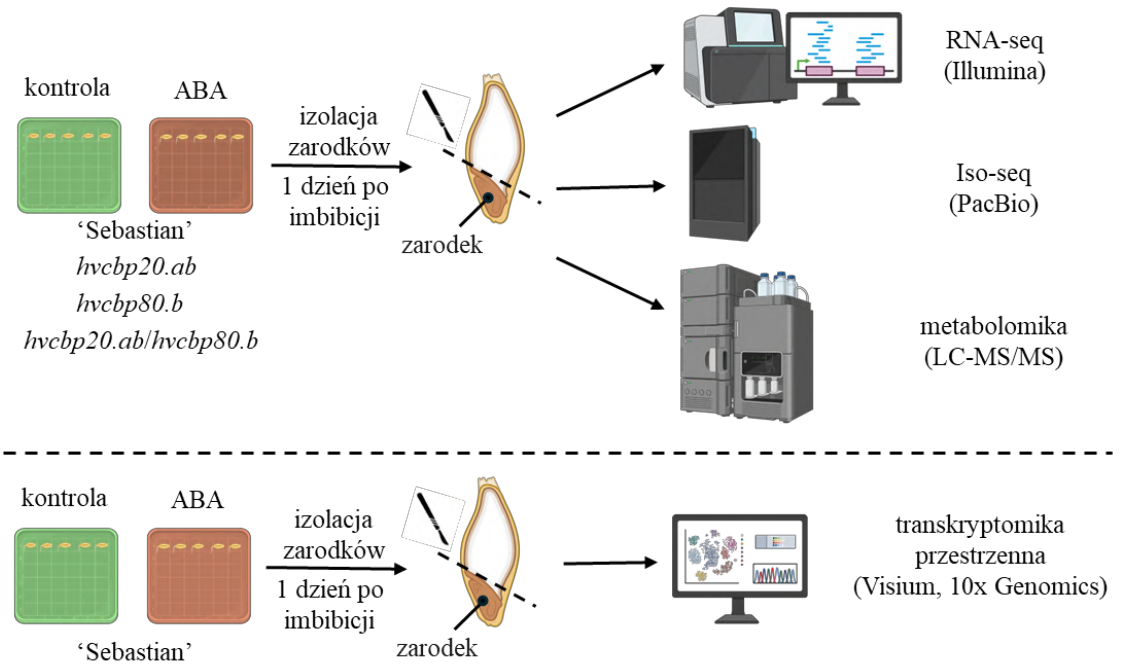
**(1) Przeprowadzono test wrażliwości na ABA w czasie kiełkowania ziarniaków.** Wrażliwość na 75 µM ABA ziarniaków *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab*/*hvcbp80.b* i odmiany rodzicielskiej 'Sebastian' oceniano na podstawie ich zdolności do kiełkowania, definiowanej jako pojawienie się korzenia zarodkowego w 1. oraz 7. dniu od zakończenia imbibicji (DAI, day after imbibition) **(P2)**. Eksperyment przeprowadzono w trzech niezależnych seriach doświadczalnych, obejmujących po trzy powtórzenia biologiczne. Każde powtórzenie biologiczne stanowiła szalka zawierająca 30 ziarniaków każdego genotypu.

**(2) Pozyskano materiał biologiczny do analiz transkryptomu, metabolomu i transkryptomiki przestrzennej**. W 1 DAI, izolowano zarodki z ziarniaków kiełkujących w warunkach kontrolnych, jak i w obecności 75 µM ABA. Z otrzymanych zarodków *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab*/*hvcbp80.b* i odmiany 'Sebastian' ekstrahowano RNA celem sekwencjonowania transkryptomu metodą krótkich odczytów Illumina **(P2, P3)** oraz długich odczytów PacBio **(P3)**, ponadto przygotowano próby do ekstrakcji metabolitów roślinnych z wykorzystaniem wysokosprawnej chromatografii cieczowej sprzężonej ze spektrometrią mas (LC-MS/MS) **(P2)**.

Z wyizolowanych zarodków odmiany 'Sebastian' przygotowano kriogeniczne skrawki tkankowe do analizy transkryptomiki przestrzennej (Visium, 10x Genomics) **(P3)**. Schemat obrazujący zastosowany układ eksperymentalny ilustruje Ryc. 3.



21:4716013696




Rycina 3. Schematyczne przedstawienie zastosowanego układu eksperymentalnego. Rycina przygotowana z wykorzystaniem BioRender (https://biorender.com/).

## 8.2 Metody

### 8.2.1 Izolacja RNA do analiz transkryptomu

Wyizolowane zarodki przechowywane w odczynniku RNAlater (Invitrogen) posłużyły do izolacji RNA, przeprowadzonej z wykorzystaniem zestawu mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific). Izolację wykonano w czterech powtórzeniach biologicznych, z których każde stanowiło 20 zarodków. Do analizy RNA-seq w technologii Illumina przygotowano 32 próby RNA, uzyskane z czterech genotypów ('Sebastian', *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b*), w czterech powtórzeniach biologicznych i dwóch warunkach traktowania (kontrola, 75 µM ABA). Parametry jakości i czystości RNA sprawdzano przy użyciu spektrofotometru NanoDrop (NanoDrop Technologies, Wilmington, USA) oraz analizatora Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA). Próby RNA (30 µl) zostały przesłane do firmy Macrogen Inc. (Korea Południowa) w celu przygotowania bibliotek cDNA metodą TruSeq Stranded mRNA oraz przeprowadzenia sekwencjonowania na platformie NovaSeq6000 (Illumina, PE, 2×150 bp, ~40 mln odczytów na próbkę). Do sekwencjonowania transkryptomu w technologii Iso-Seq (PacBio), przygotowano osiem puli RNA (40 µl), wykorzystując RNA uzyskane z tych samych zarodków, co w przypadku sekwencjonowania RNA-seq Illumina. RNA z czterech powtórzeń



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biologicznych zostało połączone w jedną pulę, tak aby każda z nich odpowiadała jednemu wariantowi eksperymentalnemu (genotyp × traktowanie). Pule RNA przesłano do firmy Macrogen Inc. (Korea Południowa), gdzie przygotowano biblioteki SMRTbell Iso-Seq zgodnie z zaleceniami producenta, a następnie przeprowadzono sekwencjonowanie na platformie PacBio Sequel II.

### 8.2.2 Analiza transkryptomu

W skrócie, surowe odczyty uzyskane z sekwencjonowania Illumina poddano kontroli jakości przy użyciu oprogramowania FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Następnie usunięto adaptory, przycięto końce o niskiej jakości za pomocą narzędzia cutadapt (Martin, 2011). Ponownie wykonano kontrolę jakości FastQC. Otrzymane odczyty zmapowano do transkryptomu referencyjnego jęczmienia BaRTv2.18 przy użyciu narzędzia Kallisto (Bray *i in.*, 2016; Coulter *i in.*, 2022). Z kolei, we współpracy z The James Hutton Institute (Dundee, Szkocja) surowe odczyty Iso-Seq (PacBio) zostały przetworzone zgodnie z potokiem analitycznym RTDBox (https://rtdbox.hutton.ac.uk), integrując je z danymi RNA-seq (Illumina) w celu opracowania referencyjnego genotypowo-specyficznego transkryptomu jęczmienia dla kiełkujących zarodków BarkeRTD (Barke Reference Transcriptome Dataset). Surowe dane RNA-seq zdeponowano w bazie EMBL-EBI (European Bioinformatics Institute) w repozytorium ArrayExpress (https://www.ebi.ac.uk/) pod numerem dostępowym E-MTAB-13989.

Zmapowane odczyty RNA-seq (Illumina) zostały zliczone i znormalizowane do wartości TPM (transcripts per million) przy użyciu oprogramowania Kallisto (Bray *i in.*, 2016). Następnie znormalizowane dane wykorzystano do analizy różnicowej ekspresji genów przy pomocy pakietu limma-voom, działającego w ramach potoku analitycznego 3D RNA-seq (Law *i in.*, 2014; Guo *i in.*, 2021). Umożliwiło to przeprowadzenie różnicowej analizy ekspresji zarówno na poziomie genów (DEG; differentially expressed genes), jak i transkryptów (DET; differentially expressed transcripts), a także identyfikację różnicowego alternatywnego składania genów (DAS; differential alternative splicing) oraz różnicowego wykorzystywania transkryptów (DTU; differential transcript usage) (Ryc. 4).



23:7507129885

Analizy przeprowadzono w grupach kontrastowych:

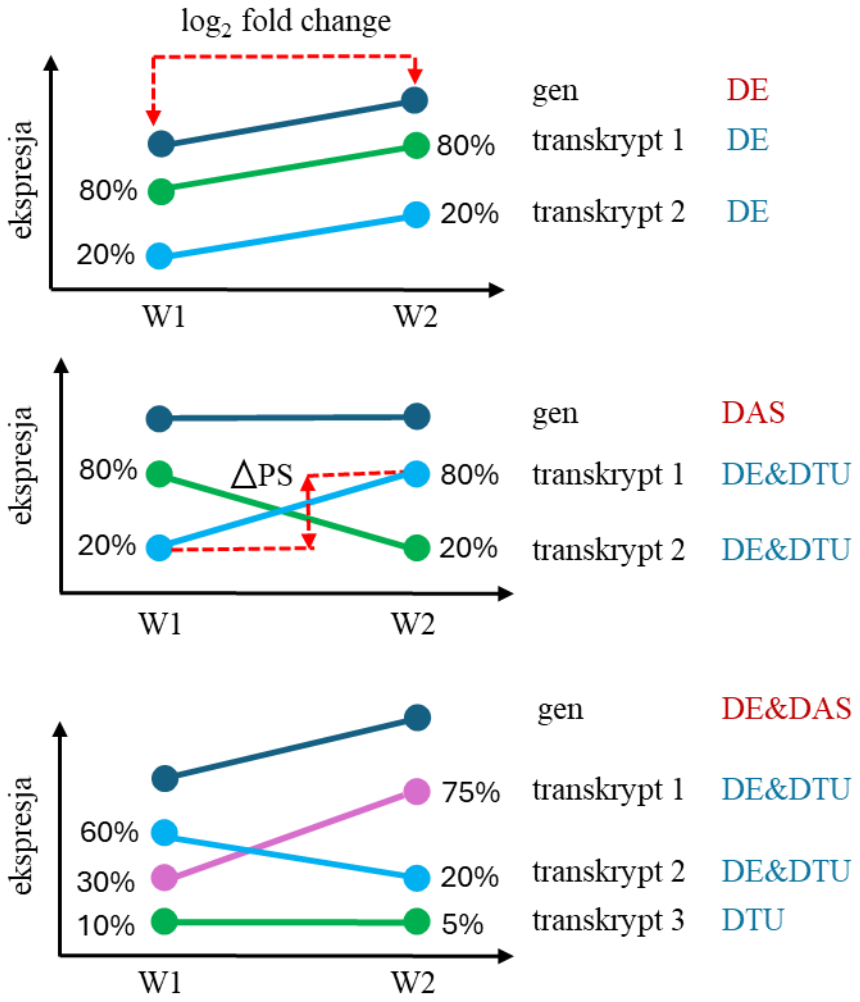
1. Mutanty *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab*/*hvcbp80.b* w warunkach kontrolnych w stosunku do odmiany ‘Sebastian’ w warunkach kontrolnych.
2. Mutanty *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab*/*hvcbp80.b* i odmiana ‘Sebastian’ po traktowaniu ABA w stosunku do warunków kontrolnych.

Różnicową ekspresję genów (DEG) i transkryptów (DET) określa się na podstawie zmiany poziomu ich ekspresji pomiędzy warunkami eksperymentalnymi, mierzonej jako wartość $\log_2$FC ($\log_2$ fold change) oraz przy skorygowanej wartości p-value W niniejszej pracy, za statystycznie istotne uznawano zmiany przy $\log_2\text{FC} \geq 1{,}5$ lub $\leq -1{,}5$ oraz skorygowanym p-value $< 0{,}01$. DAS obejmuje geny, w których w danej grupie kontrastowej dochodzi do zmian proporcji co najmniej dwóch wariantów transkryptów na skutek alternatywnego splicingu, mierzonego jako zmiana procentowego udziału izoformy ΔPS (percent spliced). W pracy za istotne statystycznie uznawano zmiany przy $\Delta\text{PS} \geq 0{,}5$. Natomiast DTU określa się na podstawie zmian we względnych proporcjach poszczególnych izoform transkryptów wytwarzanych z jednego genu pomiędzy różnymi warunkami eksperymentalnymi, niezależnie od tego, czy całkowity poziom ekspresji genu ulega zmianie. DTU występuje wtedy, gdy rozkład ilości transkryptów danego genu ulega przekształceniu, wtedy niektóre izoformy stają się w większym, a inne w mniejszym stopniu reprezentowane w różnych stanach biologicznych lub pod wpływem różnych czynników. Zjawisko to często wynika z alternatywnego splicingu i może prowadzić do istotnych zmian funkcjonalnych, takich jak przejście między izoformami kodującymi i niekodującymi białka lub izoformami o odmiennych właściwościach biologicznych (Guo *i in.*, 2021). Transkrypt uznawano za istotnie różnicowo wykorzystywany, jeżeli spełniał kryterium skorygowanej wartości p-value oraz przekraczał ustalony próg ΔPS.

Uzyskane dane z analizy różnicowej ekspresji zostały przeanalizowane i zwizualizowane z wykorzystaniem narzędzi bioinformatycznych oraz pakietów dostępnych w środowisku RStudio (https://rstudio.com; Tabela 1).



24:7900685349




Rycina 4. Schematyczne przedstawienie kryteriów identyfikacji genów o zróżnicowanej ekspresji (DEG), transkryptów o zróżnicowanej ekspresji (DET), różnicowego alternatywnego składania genów (DAS) oraz różnicowego wykorzystywania transkryptów (DTU). Linie przedstawiają średnie zmiany ekspresji genu i jego transkryptów między dwoma warunkami (W1 i W2). Granatowa linia obrazuje całkowitą ekspresję genu, będącą sumą ekspresji poszczególnych transkryptów (linia: zielona, niebieska, fioletowa). Wartości procentowe wskazują udział danego transkryptu w całkowitej ekspresji genu. DE - różnicowa ekspresja (differential expression), ΔPS - zmiana procentowego udziału izoformy (percent spliced). Na podstawie: Guo i *in*., 2021, zmodyfikowano.



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Tabela 1. Zestawienie narzędzi i pakietów bioinformatycznych wykorzystanych w analizie danych z różnicowej ekspresji.

| **Zastosowanie** | **Nazwa narzędzia bioinformatycznego lub pakietu** |
|---|---|
| Hierarchiczne klastrowanie genów | clust https://github.com/BaselAbujamous/clust/ |
| Wyodrębnienie z analizowanych grup kontrastowych puli genów/transkryptów wspólnych oraz specyficznych oraz ich wizualizacja | Venny https://bioinfogp.cnb.csic.es/tools/venny/ |
| Generowanie map cieplnych | pheatmap https://CRAN.R-project.org/package=pheatmap |
| Analiza funkcjonalna na podstawie ontologii genów (GO enrichment analysis) | TopGO https://bioconductor.org/packages/topGO/ |
| Wizualizacja wyników analizy funkcjonalnej na podstawie ontologii genów (GO enrichment analysis) | ggplot2 https://github.com/tidyverse/ggplot2 |
| Identyfikacja wzbogaconych szlaków metabolicznych | KEGG (Kyoto Encyclopedia of Genes and Genomes) https://www.genome.jp/kegg/ |
| Wizualizacja wyników analizy wzbogacenia szlaków KEGG | clusterProfiler https://bioconductor.org/packages/clusterProfiler/ |
| Pozyskanie sekwencji promotorowych genów | BioMart https://plants.ensembl.org/biomart/martview/ |
| Predykcja czynników transkrypcyjnych i miejsc ich wiązania *in silico* | PlantRegMap https://planttfdb.gao-lab.org/ |
| Identyfikacja homologów Arabidopsis | BLAST https://plants.ensembl.org/Multi/Tools/Blast |
| Predykcja interakcji białko-białko | STRING https://string-db.org/ |
| Wizualizacji sieci interakcji białko-białko | Cytoscape https://cytoscape.org/ |
| Wizualizacja zestawów danych w formie wykresu typu UpSet | ComplexUpset https://github.com/hms-dbmi/UpSetR |



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### 8.2.3 Analiza metabolomu

Do przygotowania prób do analiz metabolomicznych, w ramach analiz w **(P2)** i **(P3)**, wyizolowane zarodki z kiełkujących ziarniaków w 1 DAI (‘Sebastian’, *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab*/*hvcbp80.b*) w warunkach kontrolnych i obecności 75 µM ABA poddano homogenizacji w ciekłym azocie, a następnie natychmiast schłodzono na lodzie w celu zahamowania aktywności enzymatycznej. Do probówek o pojemności 2ml odważano $40 \pm 1$ mg uzyskanej tkanki, po czym dodawano 1 ml mieszaniny chloroform:metanol:woda dejonizowana przygotowanej w stosunku 1:2.5:1. Próbki wytrząsano przez 15 minut w temperaturze 4 °C na wytrząsarce typu Vortex i trzymano na lodzie. Następnie próbki wirowano w temperaturze 4 °C, przy 5000 × g przez 3 minuty. Otrzymane supernatanty, zawierające zarówno metabolity polarne, jak i niepolarne, przenoszono do nowych probówek. Tak przygotowane próby przesłano do laboratorium w Aberystwyth University (Wielka Brytania), gdzie przeprowadzono dalsze etapy przygotowania prób do analiz. Próby częściowo odparowano z użyciem rotacyjnego odparowywacza Buchi Rotavapor RE120, wyposażonego w pompę próżniową Buchi V5-1 oraz łaźnię wodną Buchi 461, utrzymywaną w temperaturze 25 °C, unikając ich całkowitego wysuszenia. Następnie pobrano 100 µl ekstraktu do wykonania analiz metabolomicznych metodą chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (LC-MS/MS) (Baptista *i in.*, 2018). Eksperyment przeprowadzono w czterech powtórzeniach biologicznych. Dla każdego metabolitu obliczono podstawowe statystyki opisowe, obejmujące średnią arytmetyczną, odchylenie standardowe oraz błąd standardowy. Różnice w poziomie metabolitów pomiędzy próbkami traktowanymi ABA a próbami kontrolnymi oceniano za pomocą testu t-Studenta dla prób niezależnych. Obliczono względne zmiany poziomu metabolitów (FC; fold change) pomiędzy warunkami ABA i kontrolą. Metabolity o poziomie istotności $p \leq 0{,}05$ uznawano za istotne statystycznie.

### 8.2.4 Integracja danych transkryptomicznych i metabolomicznych oraz analiza porównawcza transkryptomów

W przypadku badań przedstawionych w **(P3)** dokonano szczegółowej analizy genów o zróżnicowanej ekspresji (DEG) w grupie kontrastowej odmiana ‘Sebastian’ po traktowaniu ABA w porównaniu do warunków kontrolnych, wyodrębnionej zgodnie z podejściem opisanym w punkcie „8.2.2 Analiza transkryptomu” niniejszej rozprawy doktorskiej.



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Zidentyfikowane geny zostały następnie wykorzystane w dwóch analizach: **(1)** do integracji z danymi metabolomicznymi z zastosowaniem analizy dyskryminacyjnej metodą częściowych najmniejszych kwadratów (PLS-DA; Partial Least Squares Discriminant Analysis), przeprowadzonej z wykorzystaniem pakietu holomics w środowisku RStudio (Munk *i in.*, 2024).
**(2)** do porównania z danymi z analizy różnicowej ekspresji genów podczas rozwoju ziarniaków jęczmienia, opublikowanymi przez Kovacik *i in*. (2024), dostępnymi w materiałach dodatkowych publikacji oraz w bazie Gene Expression Omnibus (GEO) pod numerem dostępowym GSE233316. Wspólne geny występujące w obu zestawach danych identyfikowano poprzez porównanie identyfikatorów genów odniesionych do genomu referencyjnego jęczmienia MorexV3 (Mascher *i in.*, 2021), z wykorzystaniem narzędzia Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/).

### 8.2.5 Transkryptomika przestrzenna

Procedurę przeprowadzono podążając za protokołem opisanym w pracy Peirats-Llobet *i in.* (2023). Do eksperymentu wykorzystano zarodki jęczmienia odmiany ‘Sebastian’, wyizolowane z kiełkujących ziarniaków w 1 DAI zarówno w warunkach kontrolnych, jak i z dodatkiem 75 µM ABA **(P3)**. Zarodki zatapiano w medium OCT (OPTIMAL CUTTING TEMPERATURE), zamrażano w izopentanie na suchym lodzie i przechowywano w temperaturze -80 °C. Skrawki o grubości 10 µm przygotowano w kriostacie Leica CM3050 S w temperaturze -18 °C, po czym umieszczano je na szkiełkach Visium Spatial Gene Expression (10x Genomics). Następnie skrawki utrwalano w schłodzonym metanolu, barwiono safraniną O (Sigma-Aldrich, kat. nr S8884-25G) i płukano w serii etanolowej (50%, 70%, 100%; po 1 min każda). Obrazowanie wykonano w jasnym polu mikroskopem świetlnym (Leica DS5500). RNA izolowano przy użyciu zestawu RNeasy Mini Kit (Qiagen), a jego integralność oceniano za pomocą analizatora Agilent 2100 Bioanalyzer (Agilent Technologies). W celu optymalizacji procesu permeabilizacji, szkiełka montowano w kasecie Visium i inkubowano w roztworze do permeabilizacji, zawierającym bufor 10x Exonuclease I (ThermoScientific, #EN0581), albuminę surowicy owczej BSA (10% roztwór wodny, wolny od nukleaz; Sigma-Aldrich, kat. nr 126615-25ML) oraz 2% (m/v) poliwinylopirolidonu PVP40 (Sigma-Aldrich, kat. nr PVP40-500G), w temperaturze 37 °C przez 30 minut. Szkiełko płukano w buforze 0,1× SSC (SALINE-SODIUM CITRATE), a następnie przeprowadzano permeabilizację z użyciem gotowej mieszaniny



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Permeabilization Mix™ (10x Genomics) w temperaturze 37 °C. Czas permeabilizacji został dostosowany do rodzaju zastosowanych szkiełek. Dla szkiełek optymalizacyjnych „TO" (TISSUE OPTIMIZATION) zastosowano czasy 2, 4, 6, 12, 18 oraz 24 minut, natomiast dla szkiełek „GE" (GENE EXPRESSION), przeznaczonych do analizy ekspresji genów wybrano czas 6 minut. Po tym etapie szkiełka ponownie płukano w buforze 0,1× SSC. Następnie, zgodnie z zaleceniami producenta (10x Genomics User Guide, PN-1000186, CG000239_VisiumSpatialGeneExpression_UserGuide_RevD), na każdy obszar szkiełka nanoszono mieszaninę do odwrotnej transkrypcji (reverse transcription mixture™, 10x Genomics) i inkubowano w temperaturze 53 °C przez 45 minut. W przypadku szkiełek „TO", tkankę usuwano enzymatycznie. Natomiast szkiełka „GE" poddawano dalszym etapom protokołu Visium (10x Genomics Visium Spatial Gene Expression User Guide). Sekwencjonowanie cDNA przeprowadzono w trybie paired-end (PE, 2×151 bp) na platformie Illumina NovaSeq 6000 (Macrogen, Holandia). Surowe dane RNA-seq zdeponowano w bazie EMBL-EBI (European Bioinformatics Institute) w repozytorium ArrayExpress (https://www.ebi.ac.uk/) pod numerem dostępowym E-MTAB-14835. Obróbkę surowych danych wykonano przy użyciu oprogramowania Space Ranger v3.1.0 (https://www.10xgenomics.com/support/software/space-ranger/latest), mapując odczyty do genomu referencyjnego jęczmienia MorexV3 (Mascher *i in.*, 2021). Przetworzone dane wizualizowano przy użyciu oprogramowania Loupe Browser 8 (https://www.10xgenomics.com/support/software/loupe-browser/). Analizę różnicowej ekspresji genów (DEG) przy $\log_2 FC \geq 0{,}25$ lub $\leq -0{,}25$, p-value $\leq 0{,}05$, między warunkami traktowania ABA a warunkami kontrolnymi przeprowadzono w sześciu przestrzennych klastrach tkankowych, odpowiadających strukturom w obrębie kiełkującego zarodka - pochewce liścieniowej (koleoptylu), liścieniu, mezokotylu, liściu zarodkowemu (plumuli), tarczce (scutellum) oraz korzeniu zarodkowemu.



29:7653956093

## 9. Omówienie i dyskusja najważniejszych wyników

***P2: Sybilska E.**, Collin A., Sadat Haddadi B., Mur L.A.J., Beckmann M., Guo W., Simpson C.G., Daszkowska-Golec A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (Hordeum vulgare). Scientific Reports, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9*

Aby zweryfikować postawione hipotezy badawcze **(H1-H2)** dotyczące roli kompleksu CBC (Cap-Binding Complex) w kiełkowaniu ziarniaków w obecności ABA, w pierwszym etapie badań przeprowadzono analizę, mającą na celu określenie wrażliwości podwójnego mutanta *hvcbp20.ab/hvcbp80.b* na ABA. Oceniano kiełkowanie czterech badanych genotypów jęczmienia, odmiany rodzicielskiej 'Sebastian', pojedynczych mutantów *hvcbp20.ab* i *hvcbp80.b* oraz podwójnego mutanta *hvcbp20.ab/hvcbp80.*b, w warunkach kontrolnych oraz w obecności 75 µM ABA, w 1. i 7. dniu po imbibicji (DAI, day after imbibition). Zarówno w 1 DAI jak i w 7 DAI, w warunkach kontrolnych badane genotypy kiełkowały na poziomie zbliżonym do 100%. Jednak obecność ABA już w 1 DAI hamowała kiełkowanie wszystkich genotypów, szczególnie pojedynczych mutantów *hvcbp20.ab* i *hvcbp80.b*. W 7 DAI pojedyncze mutanty kiełkowały na poziomie 40-50%, podczas gdy podwójny mutant *hvcbp20.ab/hvcbp80.b* oraz odmiana 'Sebastian' osiągały podobny, wysoki poziom kiełkowania (ok. 90%). Była to zaskakująca obserwacja, ponieważ dotychczasowe badania wskazują, że pojedyncze mutanty są wrażliwe na ABA (Hugouvieux *i in.*, 2001; Papp *i in.*, 2004; Daszkowska-Golec *i in.*, 2017). Na podstawie uzyskanych wyników, wysunięto wniosek, że podwójny mutant *hvcbp20.ab/hvcbp80.b* jest niewrażliwy na ABA na etapie kiełkowania ziarniaków, w stopniu zbliżonym do odmiany 'Sebastian', w przeciwieństwie do wrażliwych na ABA pojedynczych mutantów *hvcbp20.ab* i *hvcbp80.b*. Niespodziewany fenotyp podwójnego mutanta *hvcbp20.ab/hvcbp80.b* doprowadził do rozważań nad potencjalnymi mechanizmami molekularnymi odpowiedzialnymi za jego fenotyp zbliżony do odmiany 'Sebastian'. W literaturze znane są przypadki, w których podwójne mutacje prowadzą do rewersji fenotypu do typu dzikiego (WT; wild-type), pomimo wyraźnych zaburzeń fenotypowych obserwowanych u pojedynczych mutantów (Xiao *i in.*, 2004; Seyed Rahmani *i in.*, 2021; Guo *i in.*, 2024). Jednak na tym etapie pracy trudno jednoznacznie stwierdzić, czy zaobserwowany fenotyp *hvcbp20.ab/hvcbp80.b* wynika z działania mechanizmu



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kompensacyjnego, czy jest bezpośrednim skutkiem upośledzenia funkcjonalności kompleksu CBC. Dane sugerują, że istnieje prawdopodobieństwo, że zmutowane podjednostki kompleksu CBC mogą nadal w zmieniony sposób wchodzić ze sobą w interakcje, co mogłoby prowadzić do powstania stabilnej formy kompleksu, zdolnej do pełnienia funkcji regulacyjnych w kiełkujących ziarniakach jęczmienia w odpowiedzi na ABA.

Fenotyp podwójnego mutanta stał się punktem wyjścia do dalszych badań prowadzonych w ramach niniejszej rozprawy doktorskiej, ukierunkowanych na wyjaśnienie molekularnych przyczyn kiełkowania ziarniaków *hvcbp20.ab/hvcbp80.b* w obecności ABA. Ze względu na cechę niewrażliwości na ABA obserwowaną u podwójnego mutanta i odmiany 'Sebastian', przeprowadzono hierarchiczne klastrowanie genów, aby wśród genów o zróżnicowanej ekspresji (DEG) wyodrębnić klastry genów ulegających koekspresji u obu genotypów w odpowiedzi na ABA. Wśród 12 klastrów zidentyfikowano klaster C8, w którym geny wykazywały podwyższoną ekspresję zarówno u *hvcbp20.ab/hvcbp80.b*, jak i u odmiany 'Sebastian', podczas gdy u pojedynczych mutantów ich ekspresja była obniżona, oraz klaster C11, w którym zaobserwowano odwrotną zależność, geny ulegały zmniejszonej ekspresji u podwójnego mutanta i odmiany 'Sebastian', natomiast podwyższonej u pojedynczych mutantów. Ujawnia to specyficzny wzorzec ekspresji genów u *hvcbp20.ab/hvcbp80.b*, odmienny od pojedynczych mutantów, a częściowo zbliżony do profilu obserwowanego u odmiany 'Sebastian', co wskazuje na zachowaną regulację niektórych genów w odpowiedzi na ABA mimo mutacji w obu podjednostkach CBC. Analiza ta pozwoliła na wyodrębnienie genów potencjalnie powiązanych z niewrażliwością na ABA, a także tych, których regulacja może zachodzić niezależnie od funkcjonalnego CBC.

W kontekście wcześniejszych doniesień literaturowych wskazujących na udział kompleksu CBC w regulacji zarówno konstytutywnego, jak i alternatywnego splicingu pre-mRNA m.in. poprzez interakcje z białkiem SE (SERRATE) oraz innymi czynnikami splicingowymi, zasadne stało się zweryfikowanie hipotezy, że funkcja CBC w kiełkowaniu ziarniaków jęczmienia w odpowiedzi na ABA może być ściśle związana z kontrolą procesu alternatywnego splicingu **(H1)** (Laubinger *i in.*, 2008; Raczynska *i in.*, 2010). Przeprowadzenie analiz obejmujących zarówno geny o zróżnicowanej ekspresji (DEG) i transkrypty o zróżnicowanej ekspresji (DET), jak również różnicowo alternatywnie składane geny (DAS) oraz różnicowo wykorzystywane transkrypty (DTU), umożliwiło uchwycenie nie tylko zmian ilościowych w ekspresji genów,



31:1006942033

ale również różnic w ekspresji i strukturze izoform transkryptów, co ma kluczowe znaczenie w badaniach nad mechanizmem alternatywnego splicingu. Wyniki tych analiz wykazały, że w warunkach kontrolnych zmiany w transkryptomie wszystkich badanych genotypów były niewielkie. Natomiast traktowanie ABA spowodowało znaczący wzrost liczby genów o zróżnicowanej ekspresji (DEG), transkryptów o zróżnicowanej ekspresji (DET), różnicowo alternatywnie składanych genów (DAS), a także różnicowo wykorzystywanych transkryptów (DTU) u wszystkich genotypów, co podkreśla istotną rolę ABA jako induktora zmian ekspresji oraz wskazuje, że kompleks CBC odgrywa szczególną rolę podczas aktywacji odpowiedzi na ten fitohormon.
W porównaniu do odmiany rodzicielskiej, liczba genów o zróżnicowanej ekspresji (DEG) u podwójnego mutanta *hvcbp20.ab/hvcbp80.b* była wyższa o 14%, a liczba różnicowo alternatywnie składanych genów (DAS) o 49%. Z kolei liczba transkryptów o zróżnicowanej ekspresji (DET) była wyższa o 43%, a liczba różnicowo wykorzystywanych transkryptów (DTU) aż o 66%. W odniesieniu do *hvcbp20.ab*, wartości te były wyższe odpowiednio o 12%, 48%, 37% i 50%, natomiast w porównaniu z *hvcbp80.b* o 7%, 48%, 24% i 52%. Sugeruje to, że mechanizmy transkrypcji i alternatywnego splicingu w podwójnym mutancie *hvcbp20.ab/hvcbp80.b* wykazują zwiększoną aktywność w odpowiedzi na działanie ABA w porównaniu do pozostałych genotypów. Szczególnie istotny jest fakt, że aż 72% specyficznie różnicowo alternatywnie składanych genów (DAS) u *hvcbp20.ab/hvcbp80.b* zawiera transkrypty DTU, co wskazuje, że mutacja w obu genach kodujących podjednostki CBC w znaczący sposób wpływa na modulację proporcji w wykorzystywaniu określonych izoform transkryptów. Badania Raczyńskiej *i in*. (2010) sugerowały, że podjednostka CBP80 odgrywa dominującą rolę w regulacji splicingu alternatywnego, szczególnie w odniesieniu do pierwszego intronu, w siewkach *Arabidopsis thaliana*.
W przeciwieństwie do tych obserwacji, otrzymane wyniki wskazują, że w przypadku kiełkujących zarodków jęczmienia, w regulację alternatywnego splicingu w odpowiedzi na ABA zaangażowany jest cały kompleks CBC. Może to być wynikiem specyfiki działania kompleksu CBC w odpowiedzi na sygnał fitohormonalny, jakim jest ABA, ale także różnic gatunkowych, tkankowych lub zależności od etapu rozwojowego.
Analiza funkcjonalna na podstawie ontologii genów (GO enrichment analysis) genów o specyficznie zróżnicowanej ekspresji (DEG) o podwyższonej ekspresji u *hvcbp20.ab/hvcbp80.b* ujawniła, nadreprezentację procesów związanych z hydrolizą wiązań fosfodiestrowych kwasów nukleinowych (GO:0090305). Może to wskazywać



32:1089239713

na zwiększoną aktywność mechanizmów obróbki RNA, w tym alternatywnego splicingu, który jako proces polegający na precyzyjnym wycinaniu intronów i łączeniu eksonów w pre-mRNA, wiąże się z modyfikacją wiązań fosfodiestrowych (Lorković *i in.*, 2000; Petrillo, 2023). Co więcej, w obrębie genów o podwyższonej ekspresji u *hvcbp20.ab/hvcbp80.b* zidentyfikowano specyficznie zwiększoną ekspresję sześciu homologów czynników splicingowych *Arabidopsis thaliana*: *CDC5* (*CELL DIVISION CYCLE 5*; BaRT2v18chr5HG272090), *U2AF35A* (*U2AF SMALL SUBUNIT A*; BaRT2v18chr5HG254110), *SUA* (*SUPPRESSOR OF ABI3-5*; BaRT2v18chr3HG159600), *EMB2016* (*EMBRYO-DEFECTIVE 2016*; BaRT2v18chr7HG366170), *SF1* (*PUTATIVE BRANCHPOINT-BRIDGING PROTEIN-LIKE*; BaRT2v18chr2HG081650) oraz *RS2Z33* (*SPLICING FACTOR, ARGININE/SERINE-RICH 4*; BaRT2v18chr1HG011980). Równolegle, u podwójnego mutanta zidentyfikowano łącznie 24 czynniki transkrypcyjne (TF) o specyficznie zróżnicowanej ekspresji, co stanowiło najwyższą liczbę w porównaniu do pozostałych genotypów. Wśród nich szczególną uwagę zwróciło pięć, które według przeprowadzonych analiz *in silico*, mogą wiązać się z sekwencjami promotorowymi genów kodujących *CDC5*, *EMB2016*, *SF1*, *RS2Z33*, *U2AF35a/U2AF35b* oraz *SUA*. Sugeruje to ich potencjalny udział w regulacji ekspresji tych genów oraz pośredni wpływ na składanie pre-mRNA i kontrolę alternatywnego splicingu. Dodatkowo, analizę wzbogacono o badanie sieci interakcji białkowych *in silico* podjednostek CBC pomiędzy białkami kodowanymi przez geny o specyficznie zróżnicowanej ekspresji (DEG) i specyficznie różnicowo alternatywnie składane geny (DAS) u podwójnego mutanta *hvcbp20.ab/hvcbp80.b*. Analiza ta umożliwiła identyfikację potencjalnych fizycznych interakcji białkowych CBC związanych z regulacją maszynerii splicingowej, a obecność w sieci ważnych czynników splicingowych takich jak RS2Z33, U2AF35A i CDC5 wskazuje na możliwą funkcjonalną zależność między tymi białkami a kompleksem CBC. RS2Z33, należący do rodziny białek SR (SERINE/ARGININE-RICH), pełni istotną rolę w regulacji alternatywnego splicingu oraz odpowiedzi na stres środowiskowy (Reddy i Shad Ali, 2011). U2AF35A jest kluczowym składnikiem kompleksu U2AF, uczestniczącym w rozpoznawaniu miejsc splicingowych na końcu 3′ intronu i inicjacji montażu spliceosomu (Park *i in.*, 2017; Punzo *i in.*, 2020). Z kolei CDC5 pełni funkcję czynnika transkrypcyjnego oraz regulatora przetwarzania transkryptów pri-miRNA, oddziałując z białkami SE i DCL1 (DICER-LIKE 1), a także bierze udział w regulacji



33:1076664607

alternatywnego splicingu niektórych genów związanych z odpornością roślin na patogeny (Zhang *i in.*, 2013; Huang *i in.*, 2021; Bajczyk *i in.*, 2023).

Aby głębiej zbadać mechanizmy alternatywnego splicingu u podwójnego mutanta *hvcbp20.ab/hvcbp80.b*, w dalszej części badań przeprowadzono szczegółową analizę na poziomie transkryptów, obejmującą zarówno poziomy ekspresji poszczególnych transkryptów, jak i charakterystykę zdarzeń splicingowych prowadzących do generowania różnorodnych izoform. Również w tym przypadku, podwójny mutant charakteryzował się największą liczbą transkryptów o specyficznie zróżnicowanej ekspresji (DET), co wskazuje na wystąpienie znacznej różnorodności generowanych wariantów transkryptów. Obecność wielu transkryptów w kategoriach PTC (premature termination codon) i NMD (nonsense-mediated decay) dodatkowo uwypukla znaczenie kompleksu CBC w koordynacji alternatywnego splicingu z procesem nadzoru nad stabilnością powstających mRNA (Kalyna *i in.*, 2012). Eliminacja błędnych lub niekorzystnych izoform transkryptów może zapewniać roślinom precyzyjną regulację odpowiedzi na stres wywołany ABA. Warto zwrócić uwagę, że u podwójnego mutanta *hvcbp20.ab/hvcbp80.b* zaobserwowano specyficznie zmienioną ekspresję licznych transkryptów kodujących czynniki splicingowe (GO:0008380; RNA splicing). Szczególnie interesujące jest zwiększenie ekspresji transkryptów *SE*, ponieważ kompleks CBC współdziała z tym białkiem w regulacji alternatywnego splicingu (Laubinger *i in.*, 2008; Raczynska *i in.*, 2010). Warto przy tym zaznaczyć, że SE jest fosforylowany przez kinazę PRP4KA (Wang *i in.*, 2022), a jej transkrypt również ulega specyficznie podwyższonej ekspresji u podwójnego mutanta. Otrzymane wyniki potwierdzają hipotezę **(H1)** o zaangażowaniu kompleksu CBC w kontrolę alternatywnego splicingu, wskazując, że CBC stanowi istotny element regulacji posttranskrypcyjnej, wpływając na proces AS zarówno na poziomie genów i transkryptów, jak i poprzez możliwe oddziaływania z komponentami maszynerii splicingowej na poziomie białkowym. Dodatkowym elementem niniejszej pracy jest wygenerowanie we współpracy z The James Hutton Institute (Dundee, Szkocja) referencyjnego genotypowo-specyficznego transkryptomu jęczmienia dla kiełkujących zarodków BarkeRTD (Barke Reference Transcriptome Dataset), który może być wykorzystany w przyszłych badaniach nad mechanizmami alternatywnego splicingu, wymagającymi możliwie najdokładniejszej referencji transkryptomu dla analizowanych genotypów. W tym celu rozszerzono istniejący referencyjny zestaw transkryptomiczny BaRTv2.18, oparty na genomie odmiany ‘Barke’,



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o dane z sekwencjonowania krótkich odczytów (Illumina RNA-seq) i długich odczytów, uzyskane z kiełkujących zarodków odmiany ‘Sebastian’. Tak wzbogacony zestaw umożliwia nie tylko weryfikację wyników uzyskanych w niniejszej pracy przy użyciu technologii Illumina, ale także identyfikację dodatkowych izoform transkryptów, które mogły zostać pominięte w analizach opartych wyłącznie na danych z krótkich odczytów. Z uwagi na to, że kompleks CBC został zidentyfikowany jako negatywny regulator sygnalizacji ABA, a proces kiełkowania kontrolowany jest przez złożoną sieć interakcji między fitohormonami, w ramach niniejszej pracy postawiono drugą hipotezę badawczą **(H2)**, zgodnie z którą kompleks CBC reguluje wzajemne oddziaływania między ABA a innymi fitohormonami kontrolując zdolność ziarniaków jęczmienia do kiełkowania. Analiza funkcjonalna na podstawie ontologii genów (GO enrichment analysis) przeprowadzona dla genów o specyficznie zróżnicowanej ekspresji (DEG) u podwójnego mutanta *hvcbp20.ab/hvcbp80.b* w odpowiedzi na ABA wykazała, że wśród nadreprezentowanych procesów biologicznych wyrażanych przez geny o obniżonej ekspresji, najbardziej istotnym procesem była negatywna regulacja szlaku brasinosteroidów (GO:1900458). Sugeruje to, że u podwójnego mutanta dochodzi do deregulacji mechanizmów hamujących szlak BR, co skutkuje jego aktywacją. Szczególnie istotne wydaje się to w kontekście faktu, że ABA i BR antagonistycznie regulują różne procesy rozwojowe roślin, w tym kiełkowanie nasion (Steber i McCourt, 2001; Saini *i in.*, 2015). W niniejszej pracy odnotowano obniżoną ekspresję genów kodujących negatywne regulatory sygnalizacji BR, w tym *REM4.1* (*REMORIN*; BaRT2v18chr2HG069620), *BKI1* (*BRI1 KINASE INHIBITOR 1*; BaRT2v18chr5HG251390) oraz wcześniej niescharakteryzowanego genu *BaRT2v18chr5HG272750*. *REM4.1* stanowi bliskiego homologa genu *OsREM4.1* ryżu oraz *AtREM4.1 Arabidopsis thaliana*. Z kolei *BKI1* jest bliskim homologiem *AtBKI1*, którego rola została dobrze opisana zarówno u *Arabidopsis thaliana*, jak i u ryżu (Wang i Chory, 2006; Bücherl *i in.*, 2013; Wang *i in.*, 2014; Jiang *i in.*, 2015). Zarówno OsREM4.1, jak i OsBKI1 zakłócają tworzenie kompleksu receptora OsBRI1 z koreceptorem OsBAK1 (BRASSINOSTEROID RECEPTOR-ASSOCIATED KINASE 1), a to prowadzi do zahamowania sygnalizacji BR (Gui *i in.*, 2016). Co szczególnie ważne, szlak BR może również współdziałać z sygnalizacją ABA podczas kiełkowania nasion (Cai *i in.*, 2014; Hu i Yu, 2014). Mutanty insercyjne w genie *AtREM4.1* wykazują silną niewrażliwość na ABA podczas kiełkowania (Ransbotyn *i in.*, 2015). Sugeruje to, że REM4.1 może odgrywać rolę w integracji



35:8482177961

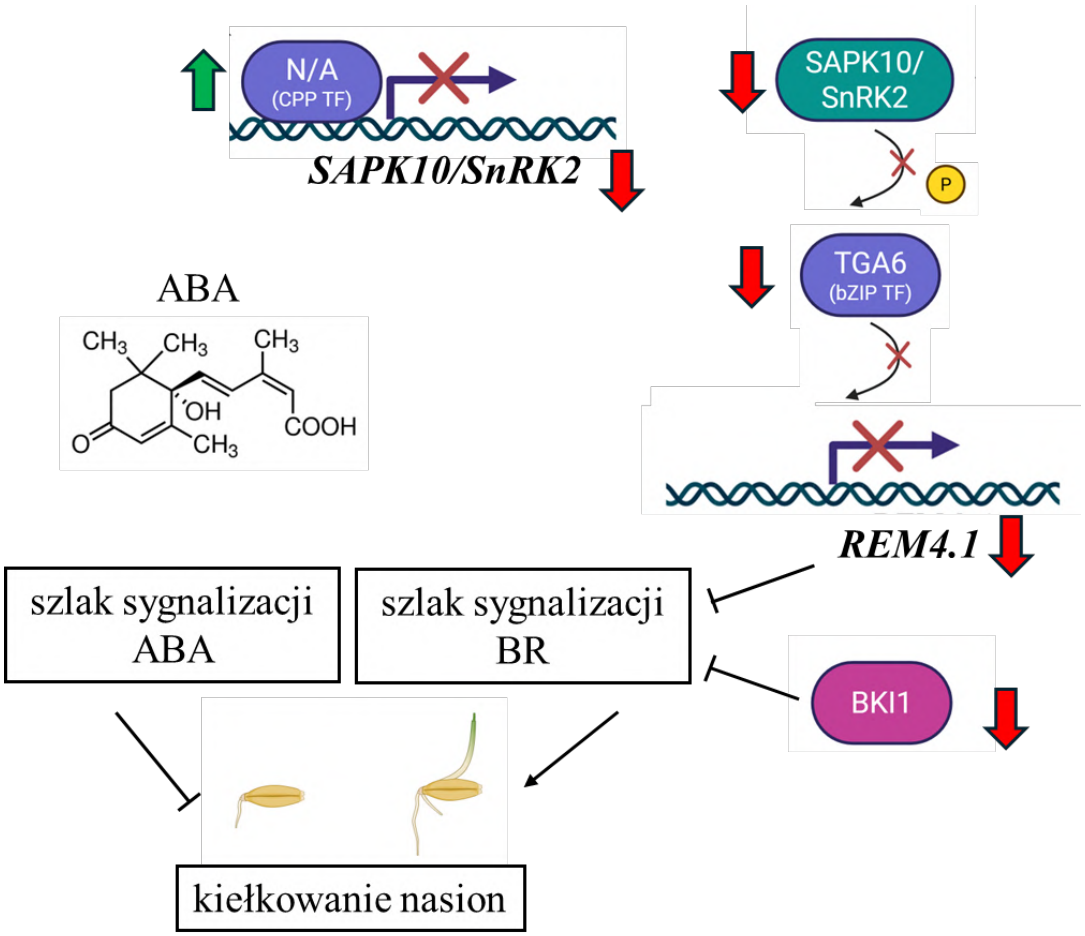
sygnałów ABA i BR na wczesnych etapach rozwoju roślin. Uwagę zwróciły również zmiany w ekspresji genów związanych ze szlakiem sygnalizacji ABA u podwójnego mutanta, w tym obniżenie ekspresji genu kodującego kinazę SAPK10 (SERINE/THREONINE-PROTEIN KINASE 10; BaRT2v18chr7HG385230). Gen ten jest homologiczny do *OsSAPK10* i wykazuje wysoki stopień podobieństwa do członków rodziny kinaz SnRK2 opisywanych u *Arabidopsis*. Dane literaturowe wskazują, że ekspresja *OsSAPK10* jest indukowana przez ABA, a jej podniesiona ekspresja prowadzi do zwiększonej wrażliwości na ten fitohormon, co podkreśla istotną rolę tej kinazy w modulacji szlaku sygnalizacji ABA (Wang *i in.*, 2020). W niniejszej pracy, na podstawie analizy *in silico* przewidywanych miejsc wiązania w obrębie sekwencji promotorowych, wskazano czynnik transkrypcyjny CPP (CYSTEINE-RICH POLYCOMB-LIKE PROTEIN) jako możliwego regulatora ekspresji homologa *REM4.1* oraz TGA6 (TGACG MOTIF-BINDING FACTOR 6) jako potencjalny czynnik kontrolujący ekspresję kinazy *SAPK10*. Na podstawie uzyskanych wyników, w niniejszej pracy zaproponowano hipotetyczny model, zgodnie w którym ABA u podwójnego mutanta *hvcbp20.ab/hvcbp80.b* indukuje ekspresję nieznanego czynnika transkrypcyjnego z rodziny CPP, który hamuje ekspresję *SAPK10*. W rezultacie dochodzi do ograniczenia fosforylacji czynnika transkrypcyjnego TGA6 i spadku ekspresji *REM4.1*, będącego negatywnym regulatorem szlaku sygnalizacji BR. Dodatkowo, ABA zmniejsza ekspresję *BKI1*, kodującego inny inhibitor sygnalizacji BR, co może sprzyjać aktywacji tego szlaku i w ten sposób wpływać na obserwowany fenotyp kiełkowania podwójnego mutanta *hvcbp20.ab/hvcbp80.b* (Ryc. 5).

Uzupełnieniem uzyskanych danych transkryptomicznych są wyniki analizy metabolomicznej, które wskazują, że mutacja w obu genach kodujących podjednostki kompleksu CBC wpływa również na regulację metabolizmu brasinosteroidów podczas kiełkowania ziarniaków w odpowiedzi na ABA. U podwójnego mutanta *hvcbp20.ab/hvcbp80.b* stwierdzono podwyższoną zawartość 6-alfa-hydroksy-6-deoksykastasteronu, metabolitu będącego prekursorem brasinolidu, kluczowego aktywnego brasinosteroidu, co sugeruje nasilenie biosyntezy BR.

Łącznie uzyskane wyniki transkryptomiczne i metabolomiczne jednoznacznie wspierają hipotezę **(H2)**, zgodnie z którą kompleks CBC odgrywa istotną rolę w regulacji interakcji pomiędzy ABA a innymi fitohormonami, wpływając tym samym na kontrolę zdolności kiełkowania ziarniaków jęczmienia.



36:4959114055




Rycina 5. Przypuszczalny schemat regulacji kiełkowania ziarniaków podwójnego mutanta *hvcbp20.ab/hvcbp80.b* integrujący sygnalizację ABA i BR. Na podstawie: Sybilska i *in*., 2024 **(P2)**, zmodyfikowano. Zielone strzałki obrazują specyficznie zwiększoną, a czerwone specyficznie obniżoną ekspresję genów u *hvcbp20.ab/hvcbp80.b* w odpowiedzi na ABA. *SAPK10/SnRK2* (*SERINE/THREONINE-PROTEIN KINASE SAPK10* / *SNF1-RELATED PROTEIN KINASE 2*), TGA6 (TGACG MOTIF-BINDING FACTOR 6), BKI1 (BRI1 KINASE INHIBITOR 1), *REM4.1* (*REMORIN*), bZIP (basic leucine zipper), CPP (CYSTEINE-RICH POLYCOMB-LIKE PROTEIN), ABA - kwas abscysynowy (abscisic acid), BR - brasinosteroidy (brassinosteroids), TF - czynnik transkrypcyjny (transcription factor), P - reszta fosforanowa.



37:2223680919

***P3: Sybilska E.**, Haddadi B.S., Mur L.A.J., Beckmann M., Hryhorowicz S., Suszyńska-Zajczyk J., Knaur M., Pławski A., Daszkowska-Golec A. (2025). Mapping the molecular signature of ABA-regulated gene expression in germinating barley embryos. BMC Plant Biology, 25(1), 619. https://doi.org/10.1186/s12870-025-06654-z*

W związku z interesującymi wynikami uzyskanymi dla zarodków podwójnego mutanta *hvcbp20.ab/hvcbp80.b* kiełkujących w 1 DAI w obecności ABA **(P1)**, przeprowadzono pogłębioną analizę tego procesu rozwojowego dla kiełkujących zarodków odmiany rodzicielskiej 'Sebastian'. Dzięki temu możliwa była bardziej szczegółowa charakterystyka mechanizmów regulacyjnych warunkujących odpowiedź na ABA na wczesnym etapie kiełkowania ziarniaków. Analiza ta miała na celu weryfikację hipotezy badawczej **(H3)**, zakładającej, że ABA indukuje specyficzne zmiany zarówno w ekspresji genów, jak i w metabolomie kiełkujących zarodków jęczmienia, pełniąc rolę sygnału molekularnego koordynującego reakcje adaptacyjne oraz interakcje fitohormonalne w odpowiedzi na stres środowiskowy. Przeprowadzona globalna analiza ekspresji genów wykazała, że w odpowiedzi na ABA obniżona zostaje ekspresja znaczącej liczby genów w kiełkujących zarodkach odmiany 'Sebastian'. Co istotne, podobny efekt zaobserwowano również w przypadku wcześniejszych badań nad podwójnym mutantem *hvcbp20.ab/hvcbp80.b* **(P1)**. Ponadto, obserwacje te są zgodne z badaniami u roślin zarówno jednoliściennych, jak i dwuliściennych (Penfield *i in.*, 2006; Yu *i in.*, 2016). Taka spójność wyników uzyskanych w różnych podejściach badawczych oraz w odniesieniu do różnych gatunków roślin, pozwala stwierdzić, że ABA działa jako represor ekspresji genów w procesie kiełkowania nasion. Aby zrozumieć sposób, w jaki ABA wpływa na regulację transkrypcji, przeprowadzono analizę *in silico* w celu identyfikacji czynników transkrypcyjnych (TF) potencjalnie zaangażowanych w kontrolę ekspresji genów odpowiedzi na ten fitohormon. Zebrane dane wskazują na istotny udział 23 TF w regulacji ekspresji genów w zarodkach podczas kiełkowania, które mają miejsca wiązania w obrębie sekwencji promotorowych aż 65% wszystkich zidentyfikowanych genów o zróżnicowanej ekspresji (DEG). Wśród nich szczególną uwagę zwrócono na czynniki transkrypcyjne ABI3 (ABSCISIC ACID INSENSITIVE 3; BaRT2v18chr3HG161790), AREB3 (ABA-RESPONSIVE ELEMENT BINDING PROTEIN 3; BaRT2v18chr1HG033690) i ABF3 (ABRE-BINDING FACTOR 3; BaRT2v18chr3HG156370). Te TF są dobrze znane ze swojej zdolności do bezpośredniego wiązania się z cis-regulacyjnymi elementami ABRE (ABA-



38:8239134034

Responsive Elements), obecnymi w promotorach genów zależnych od ABA. Dzięki temu precyzyjnie kontrolują transkrypcję genów zaangażowanych zarówno w reakcje adaptacyjne na stres abiotyczny, jak i w regulację procesu kiełkowania (Choi *i in.*, 2000; Finkelstein *i in.*, 2005; Fujita *i in.*, 2013; Yoshida *i in.*, 2014). Przeprowadzona analiza funkcjonalna na podstawie ontologii genów (GO enrichment analysis) umożliwiła identyfikację nadreprezentowanych procesów biologicznych, wyrażanych przez geny o zróżnicowanej ekspresji (DEG), regulowanych przez ABA oraz wskazała, które z nich są aktywowane, a które ulegają represji w odpowiedzi na działanie tego fitohormonu. Wykazano, że ABA hamuje ekspresję genów związanych z procesami metabolicznymi i strukturalnymi, takimi jak modyfikacja ściany komórkowej, metabolizm hemicelulozy i innych polisacharydów, odpowiedź na stres oksydacyjny oraz biosynteza fenylopropanoidów, co może sugerować ograniczenie wzrostu i aktywności metabolicznej w zarodkach jęczmienia. Z drugiej strony, ABA indukuje ekspresję genów zaangażowanych w liczne procesy związane z reakcjami adaptacyjnymi do niekorzystnych warunków środowiskowych. Co istotne, procesy biologiczne związane z odpowiedzią na ABA (GO:0009737) były wyraźnie stymulowane, a to wskazuje na uruchomienie szlaku sygnalizacji tego fitohormonu. Jest to zgodne z dobrze udokumentowanym mechanizmem, według którego stresy abiotyczne prowadzą do aktywacji sygnalizacji ABA, inicjując kaskadę molekularnych odpowiedzi adaptacyjnych (Fujita *i in.*, 2013; Ng *i in.*, 2014; Yoshida *i in.*, 2014; Chen *i in.*, 2020). Zaobserwowane zmiany podkreślają kluczową rolę ABA jako molekularnego przełącznika, który w warunkach stresu abiotycznego inicjuje przeprogramowanie aktywności komórkowej z procesów wzrostowych na mechanizmy sprzyjające przetrwaniu, co pozostaje w pełnej zgodzie z hipotezą **(H3)**. Analiza ścieżek metabolicznych z wykorzystaniem bazy danych KEGG (Kyoto Encyclopedia of Genes and Genomes) potwierdziła uzyskane wyniki analizy funkcjonalnej na podstawie ontologii genów (GO enrichment analysis), a ponadto wykazała, że traktowanie ABA znacząco intensyfikuje procesy transdukcji sygnałów fitohormonów oraz wpływa na regulację ekspresji genów ze szlaków związanych z ich biosyntezą. W obrębie tych ścieżek zmiany ekspresji dotyczyły genów związanych z sygnalizacją auksyny (AUX), kwasu jasmonowego (JA), giberelin (GA), etylenu (ET), cytokinin (CTK) oraz kwasu salicylowego (SA), a także genów uczestniczących w szlakach biosyntezy JA, brasinosteroidów (BR), diterpenoidów (w tym GA), ET, zeatyny oraz ścieżki biosyntezy kwasu indolilo-3-octowego (IAA) z tryptofanu. Zaobserwowano również, że ABA



39:8715626656

reguluje ekspresję genów własnego szlaku sygnałowego, takich jak geny kodujące fosfatazy PP2C (BaRT2v18chr3HG142490; BaRT2v18chr1HG046520; BaRT2v18chr2HG049520; BaRT2v18chr3HG157400; BaRT2v18chr3HG138810), kinazy SnRK2 (BaRT2v18chr1HG037480; BaRT2v18chr1HG026070; BaRT2v18chr4HG182300) oraz czynniki transkrypcyjne ABF/AREB (BaRT2v18chr3HG156370; BaRT2v18chr1HG033690). Jednak aby chronić komórkę przed nadmierną stymulacją fitohormonalną, odpowiedź na sygnał ABA może uruchamiać mechanizm sprzężenia zwrotnego (Merlot *i in.*, 2001; Santiago *i in.*, 2009). Wyniki uzyskane w niniejszej pracy wskazują na możliwą aktywację tego mechanizmu, co sugeruje obniżona ekspresja genu kodującego receptor PYL (BaRT2v18chr1HG034770) przy jednoczesnym wzroście ekspresji genów kodujących fosfatazy PP2C (BaRT2v18chr3HG142490, BaRT2v18chr1HG046520, BaRT2v18chr2HG049520, BaRT2v18chr3HG157400, BaRT2v18chr3HG138810). Zaobserwowano również, że traktowanie ABA doprowadziło do aktywacji jego własnego szlaku biosyntezy, na co wskazuje zwiększona ekspresja genów związanych z biosyntezą ABA, takich jak *NCED* (*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE*; BaRT2v18chr5HG223780) oraz dwóch genów adnotowanych w KEGG jako kodujące beta-karoten 3-hydroksylazę (*BETA-CAROTENE 3-HYDROXYLASE*; BaRT2v18chr2HG094980, BaRT2v18chr4HG215920). Należy podkreślić, że wyniki z analizy metabolomu kiełkujących zarodków wykazały aż trzykrotny wzrost poziomu endogennego ABA, który związany był ze zwiększoną akumulacją jego prekursorów, takich jak β-karoten i wiolaksantyna, przy jednoczesnym obniżeniu poziomu kwasu fazeinowego, głównego produktu katabolizmu ABA. Jest to zgodne z dotychczasową wiedzą, wskazującą na biosyntezę ABA w odpowiedzi roślin na stresowe warunki środowiskowe (Seo *i in.*, 2006; Frey *i in.*, 2012; Chen *i in.*, 2020; Sano i Marion-Poll, 2021). Łącznie uzyskane wyniki sugerują, że indukowany przez ABA sygnał jest wzmacniany przez równoległą intensyfikację procesu biosyntezy tego fitohormonu wskazując na skoordynowaną regulację tych szlaków na poziomie zarówno transkrypcyjnym, jak i metabolomicznym. Kluczowym aspektem dalszych analiz, mających na celu weryfikację hipotezy **(H3)**, była integracja danych transkryptomicznych i metabolomicznych. Analiza ta ujawniła wysoką korelację pomiędzy tymi zbiorami danych (r = 0,99), co wskazuje na ścisłą współzależność między transkryptomem a metabolomem kiełkujących zarodków w obecności ABA. W badaniach wyodrębniono 32 metabolity, które powiązano z 35 genami o zróżnicowanej



40:8567826237

ekspresji (DEG). Ponadto, wykazano, że zmiany w poziomie ABA pozytywnie korelują z ekspresją genów szlaku sygnalizacyjnego ABA, takich jak *SnRK2* (BaRT2v18chr4HG182300), *ABF* (BaRT2v18chr1HG033690) i *PP2C* (BaRT2v18chr3HG138810). Zaobserwowano również korelacje pomiędzy poziomami ABA a ekspresją genów powiązanych z działaniem giberelin (GA), jasmonianów (JA), auksyn (AUX), etylenu (ET) oraz kwasu salicylowego (SA). W przypadku giberelin, mimo dobrze udokumentowanej w literaturze antagonistycznej roli ABA i GA w kiełkowaniu, gdzie ABA działa jako inhibitor, a GA jako promotor tego procesu, w niniejszych badaniach odnotowano dodatnią korelację pomiędzy ABA a ekspresją receptora *GID1* (*GIBBERELLIN INSENSITIVE DWARF 1*; BaRT2v18chr1HG028980). Taki wynik może odzwierciedlać wczesną aktywację sygnalizacji GA, zapewniając tym samym płynne przejście od fazy hamowania do ponownego rozpoczęcia procesu kiełkowania po ustąpieniu stresu (Seo *i in.*, 2006; Shu *i in.*, 2016; Ali *i in.*, 2022). Jednocześnie, odnotowane w pracy obniżenie poziomu aktywnych form GA sugeruje, że katabolizm giberelin dominuje nad ich biosyntezą, mimo zwiększonej transkrypcji genów kodujących enzymy szlaku biosyntetycznego. Natomiast w przypadku jasmonianów, zależność pomiędzy ABA i JA pozostaje niejednoznaczna. Pozytywną korelację zaobserwowano pomiędzy poziomem ABA a ekspresją genu *COI1 (CORONANTINE INSENSITIVE 1;* BaRT2v18chr1HG036610), będącego kluczowym elementem aktywującym szlak sygnalizacji JA (Xu *i in.*, 2024). Choć literatura wskazuje na synergizm działania JA i ABA, istnieją również doniesienia sugerujące ich wzajemny antagonizm, który może być zależny od gatunku (Dave *i in.*, 2011; Jacobsen *i in.*, 2013; De Ollas i Dodd, 2016; Verma *i in.*, 2016). W niniejszej pracy, traktowanie ABA doprowadziło do wyraźnego spadku poziomu jasmonianów w kiełkujących zarodkach, prawdopodobnie z powodu ograniczenia dostępności jego prekursorów takich jak kwas linolenowy, kwas 13-hydroperoksyoktatryenowy (13-HPOTE) i kwas 12-oksoksofitodienowy (OPDA). Podobnie interakcje ABA z auksyną mają złożony charakter. Niektóre dane literaturowe wskazują, że auksyna zwiększa poziom ABA, co opóźnia proces kiełkowania (Park *i in.*, 2011; Shuai *i in.*, 2017). Natomiast zależność między ABA a auksyną może różnić się w zależności od dawki (Belin *i in.*, 2009). Choć w otrzymanych wynikach przeważała negatywna korelacja ABA z ekspresją genów związanych z biosyntezą, transportem, sygnalizacją i wczesną odpowiedzią na auksynę, nie odnotowano istotnych zmian w poziomach metabolitów auksyny po traktowaniu ABA. W kontekście interakcji ABA z etylenem zaobserwowano dodatnią korelację



41:1000704730

między poziomem ABA a ekspresją *EIN3 (ETHYLENE-INSENSITIVE 3;* BaRT2v18chr2HG086440) i *ERS2* (*ETHYLENE RESPONSE SENSOR 2*; *BaRT2v18chr6HG314730)*. Etylen może łagodzić działanie ABA i promować kiełkowanie, choć dane dotyczące jego wpływu są niejednoznaczne, a mutacje w genach szlaku sygnalizacji ET mogą zwiększać wrażliwość na ABA lub nie wykazywać takiego efektu (Ghassemian *i in.*, 2000; Chiwocha *i in.*, 2005; Subbiah i Reddy, 2010). Z kolei opisywana w literaturze antagonistyczna zależność między ABA a salicylanami, może potencjalnie wyjaśniać zaobserwowaną negatywną korelację pomiędzy poziomem ABA a ekspresją genu *PR1* (*PATHOGENESIS-RELATED PROTEIN 1*; BaRT2v18chr5HG244050) (Nishimura *i in.*, 2009). W świetle uzyskanych wyników, obejmujących zarówno poziom transkryptomiczny, jak i metabolomiczny, zasadne jest przyjęcie hipotezy **(H3)**, zakładającej, że ABA poprzez interakcje z innymi fitohormonami uczestniczy w precyzyjnej regulacji procesu kiełkowania ziarniaków jęczmienia.

Zważywszy, że rola ABA nie ogranicza się wyłącznie do odpowiedzi na stres abiotyczny, ale obejmuje również kontrolę procesów rozwojowych, przeprowadzono analizę mającą na celu wyodrębnienie genów indukowanych wyłącznie przez traktowanie ABA od tych, które ulegają ekspresji tylko podczas rozwoju ziarniaków jęczmienia (Qin *i in.*, 2021; Zhang *i in.*, 2021; Daszkowska-Golec, 2022). Porównanie wygenerowanych w ramach niniejszej pracy danych transkryptomicznych kiełkujących zarodków traktowanych ABA z ogólnodostępnymi danymi RNA-seq trzech różnych tkanek ziarniaka jęczmienia, pochodzącymi z badań Kovacik *i in*. (2024), pozwoliło na uzyskanie puli genów specyficznych dla traktowania ABA, a także genów wspólnych z każdą z analizowanych tkanek (zarodkiem, bielmem i tkanką macierzystą ziarniaka). W niniejszej pracy, zidentyfikowano 1586 genów o zróżnicowanej ekspresji (DEG) zależnych wyłącznie od traktowania ABA w kiełkującym zarodku. Analiza funkcjonalna na podstawie ontologii (GO enrichment analysis) tych genów wykazała, że są one istotnie powiązane z procesami organizacji ściany komórkowej, modyfikacjami struktury komórek oraz odpowiedzią na stres, w tym stres oksydacyjny. Innym ciekawym wynikiem było stwierdzenie, że spośród genów ulegających ekspresji w kiełkujących zarodkach traktowanych ABA, 2035 genów o zróżnicowanej ekspresji (DEG) wykazuje ekspresję również w zarodkach podczas rozwoju, co sugeruje obecność konserwatywnych mechanizmów regulowanych przez ABA, istotnych zarówno w odpowiedzi na stres środowiskowy, jak i w kontroli procesów rozwojowych. Warto podkreślić,



42:3842060036

że przeprowadzona analiza porównawcza obejmowała dwa różne genotypy jęczmienia. Dane transkryptomiczne opublikowane przez Kovacik *i in*. (2024) zostały wygenerowane dla jęczmienia odmiany ‘Morex’, natomiast badania przedstawione w niniejszej pracy przeprowadzono na odmianie ‘Sebastian’. Uwzględnienie w analizie dwóch różnych genotypów nadaje otrzymanym wynikom dodatkową wartość, ponieważ mogą one odzwierciedlać bardziej uniwersalne mechanizmy regulacyjne zachodzące w zarodkach jęczmienia.

W kolejnym etapie badania przystąpiono do weryfikacji hipotezy **(H4)**, zgodnie z którą ekspresja genów regulowanych przez ABA wykazuje tkankową specyficzność w różnych obszarach kiełkujących zarodków jęczmienia. W tym celu wykorzystano technologię transkryptomiki przestrzennej Visium (10x Genomics), która łączy wysokoprzepustowe sekwencjonowanie RNA z precyzyjnym określeniem lokalizacji ekspresji genów w tkankach, przy jednoczesnym zachowaniu architektury przestrzennej analizowanego materiału biologicznego. Dzięki tej metodzie możliwe było szczegółowe ustalenie profilu transkryptomicznego sześciu wyodrębnionych regionów tkankowych w obrębie kiełkującego zarodka jęczmienia: pochewki liścieniowej (koleoptylu), liścienia, mezokotylu, liścia zarodkowego (plumuli), tarczki (scutellum) oraz korzenia zarodkowego. W wyniku przeprowadzonych analiz udało się ustalić przestrzenne zróżnicowanie ekspresji 49 genów w poszczególnych tkankach zarodka. Geny te pochodziły z puli 1586 genów o zróżnicowanej ekspresji (DEG), uprzednio zidentyfikowanych na podstawie analizy RNA-seq jako specyficznie indukowane w odpowiedzi na egzogenny ABA. Analiza ujawniła, że koleoptyl wykazuje najwyższą aktywność transkrypcyjną spośród wszystkich badanych tkanek, co podkreśla jego szczególną rolę w odpowiedzi na ABA na wczesnym etapie kiełkowania. W przypadku 49 genów o przypisanej lokalizacji tkankowej, ekspresję w koleoptylu stwierdzono dla 30 genów, z czego 14 ulegało ekspresji wyłącznie w tej tkance. Wyniki te sugerują, że koleoptyl może pełnić dotychczas nieopisaną rolę jako tkanka istotna dla przetwarzania sygnałów ABA podczas wczesnych etapów kiełkowania ziarniaków. W koleoptylu odnotowano specyficznie podwyższoną ekspresję genu BaRT2v18chr7HG337790, kodującego GDSL-esterazę/lipazę. Wiadomo, że GDSL-lipazy uczestniczą w przebudowie kutyny, kluczowego składnika kutykuli, co pozwala roślinie dostosowywać właściwości powierzchniowe do warunków środowiskowych, w szczególności ograniczając utratę wody (Yeats i Rose, 2013; Shen *i in.*, 2022). Podobną funkcję pełni gen *GER1* (*GDSL CONTAINING ENZYME RICE 1*) u ryżu,



43:1016778442

kodujący enzym o aktywności GDSL-lipazy, którego ekspresja jest silnie indukowana przez światło oraz kwas jasmonowy. Transkrypt *GER1* wykazuje wysoki poziom akumulacji w koleoptylu, co wskazuje na jego potencjalną rolę w przebudowie kutykuli oraz adaptacji tkanki do zmiennych warunków środowiskowych w fazie wschodów (Riemann *i in.*, 2007). W związku z tym, uzyskane wyniki mogą wskazywać na istotną rolę ABA w remodelowaniu powierzchni koleoptylu w odpowiedzi na stres abiotyczny. Równocześnie odnotowano specyficznie podwyższoną ekspresję genu BaRT2v18chr3HG155900, adnotowanego jako *PAM2 domain-containing protein*/*EARLY RESPONSIVE TO DEHYDRATION 15* (*ERD15*). Gen ten koduje białko z konserwowanym motywem PAM2 (POLY(A)-BINDING PROTEIN-INTERACTING MOTIF 2), umożliwiającym interakcję z poli(A)-wiążącymi białkami PABP (POLY(A)-BINDING PROTEIN). Funkcja *ERD15* może być związana z regulacją posttranskrypcyjną, poprzez oddziaływanie na stabilność i translację mRNA (Aalto *i in.*, 2012). Co istotne, *ERD15* został wcześniej scharakteryzowany jako negatywny regulator szlaku sygnałowego ABA (Kariola *i in.*, 2006). Mając to na uwadze, można przypuszczać, że specyficznie zwiększona ekspresja tego genu w koleoptylu ogranicza nadmierną reakcję na ten fitohormon. Łącznie sugeruje to, że odpowiedź na ABA w koleoptylu obejmuje zarówno fizyczne wzmocnienie bariery kutykularnej, jak i równoległą regulację ekspresji genów na poziomie posttranskrypcyjnym, umożliwiającą precyzyjne dostrojenie odpowiedzi na ABA.

Zaobserwowana w niniejszej pracy zróżnicowana lokalizacja ekspresji genów zależnych od ABA w obrębie tkanek zarodkowych stanowi bezpośrednie potwierdzenie hipotezy **(H4)**, wskazując na obecność precyzyjnych, przestrzennie zorganizowanych mechanizmów regulacyjnych aktywowanych przez ABA w zarodkach jęczmienia.



44:9542029140

## 10. Wnioski

1. Obserwowane zniesienie wrażliwości na ABA podczas kiełkowania ziarniaków u podwójnego mutanta *hvcbp20.ab/hvcbp80.b*, w przeciwieństwie do wyraźnej wrażliwości pojedynczych mutantów *hvcbp20.ab* i *hvcbp80.b*, sugeruje działanie mechanizmu genetycznej kompensacji pomiędzy genami kodującymi podjednostki kompleksu CBC.
2. Alternatywny splicing jest kluczowym mechanizmem, za pośrednictwem którego kompleks CBC moduluje transkryptom i reguluje proces kiełkowania ziarniaków w odpowiedzi na sygnał ABA.
3. Możliwe, że kompleks CBC reguluje alternatywny splicing w odpowiedzi na ABA poprzez fizyczne oddziaływanie z komponentami aparatu splicingowego, modyfikując jego skład i aktywność w kiełkujących zarodkach.
4. Na podstawie wyników analiz przeprowadzonych dla podwójnego mutanta *hvcbp20.ab/hvcbp80.b* w odpowiedzi na ABA, można przypuszczać, że kompleks CBC odgrywa rolę w regulacji kiełkowania ziarniaków poprzez integrację sygnalizacji ABA z sygnalizacją i metabolizmem brasinosteroidów.
5. Egzogenny ABA reguluje kiełkowanie ziarniaków jęczmienia zwyczajnego, działając głównie jako represor ekspresji genów oraz poprzez uruchamianie złożonych, wzajemnie powiązanych sieci regulatorowych fitohormonów.
6. ABA reguluje ekspresję wspólnego zestawu genów dla stresu abiotycznego na etapie kiełkowania, jak i podczas rozwoju zarodkowego, co sugeruje istnienie uniwersalnych mechanizmów działania tego fitohormonu w różnych kontekstach fizjologicznych.
7. Odpowiedź na egzogenny ABA w kiełkujących zarodkach jęczmienia odmiany ‘Sebastian’ ma charakter przestrzennie zróżnicowany, co przejawia się specyficzną ekspresją genów w poszczególnych tkankach zarodkowych, takich jak pochewka liścieniowa (koleoptyl), liścień, zawiązek pędu (plumula), tarczka (scutellum), mezokotyl oraz korzeń zarodkowy.



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EDITED BY
Yong Xiang,
Agricultural Genomics Institute at Shenzhen (CAAS), China

REVIEWED BY
Debatosh Das,
University of Missouri, United States
Fanli Meng,
Northeast Agricultural University, China

*CORRESPONDENCE
Agata Daszkowska-Golec
agata.daszkowska@us.edu.pl

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# Alternative splicing in ABA signaling during seed germination

Ewa Sybilska and Agata Daszkowska-Golec*

Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Katowice, Poland

Seed germination is an essential step in a plant's life cycle. It is controlled by complex physiological, biochemical, and molecular mechanisms and external factors. Alternative splicing (AS) is a co-transcriptional mechanism that regulates gene expression and produces multiple mRNA variants from a single gene to modulate transcriptome diversity. However, little is known about the effect of AS on the function of generated protein isoforms. The latest reports indicate that alternative splicing (AS), the relevant mechanism controlling gene expression, plays a significant role in abscisic acid (ABA) signaling. In this review, we present the current state of the art about the identified AS regulators and the ABA-related changes in AS during seed germination. We show how they are connected with the ABA signaling and the seed germination process. We also discuss changes in the structure of the generated AS isoforms and their impact on the functionality of the generated proteins. Also, we point out that the advances in sequencing technology allow for a better explanation of the role of AS in gene regulation by more accurate detection of AS events and identification of full-length splicing isoforms.

KEYWORDS

**alternative splicing, abscisic acid, seed germination, protein isoforms, splice variant, splicing factors, ABA signaling**

## Role and molecular mechanism of alternative splicing

Alternative splicing (AS) is a significant gene regulatory process in eukaryotic organisms. As a result of AS, many transcripts are produced from one gene, which can be translated into various proteins. The produced proteins can have similar or different functions in cells and vary in their properties (Verta and Jacobs, 2022). All of this makes AS contribute to increasing transcriptome and proteome diversity (Pan et al., 2008; Nilsen and Graveley, 2010). The splicing occurs in the nucleus during transcription to pre-mRNA and is catalyzed by the spliceosome. The spliceosome is a big complex composed of proteins and RNA. It builds five small nuclear ribonucleoproteins (snRNPs), namely U1, U2, U4, U5, and U6, and many other proteins required for the proper splicing process (Reddy,



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2007; Gehring and Roignant, 2021). In two transesterification reactions catalyzed by splicosome, introns are removed, and exons are linked. In several steps, the spliceosome recognizes conserved sequences at exon-intron junctions and undergoes precise assembly and remodeling (Meyer et al., 2015) (Figure 1A). The spliceosome functions through numerous RNA and protein interactions and its activity is coordinated by multiple *cis*-acting elements and *trans*-acting factors (Punzo et al., 2020; Lin and Zhu, 2021) (Figure 1B). The constitutive and alternative splicing is distinguished. AS events can be grouped into five general splicing events such as intron retention (IR), exon skipping (ES), mutually exclusive exons, alternative 5′ donor sites (A5′SS) or alternative 3′ acceptor sites (A3′SS) (Kathare and Huq, 2021) (Figure 2). All introns are excised from pre-mRNA in constitutive splicing, and mature mRNA is assembled from exons. This produces one type of mRNA and generates only one type of protein. While in alternative splicing, introns and exons are removed or included from pre-mRNA in various ways, producing many mRNA isoforms. During exon skipping, the exon is removed from the pre-mRNA together with introns. The intron retention appears when both 5′ and 3′ ends of the specific intron are wrongly recognized, producing mRNA containing that additional intronic sequence. In mutually exclusive exons, only one exon from the cluster of exons remains in the mRNA, while the others are spliced out. When an alternative 5′ splicing site is generated, called a donor site as well, it causes cutting out of the fragment of exon from its 3′ end and nearby intron. The generation of an alternative splice site 3′, which is called the acceptor site as well, causes the cutting out of the intron with the 5′ fragment of the downstream exon (Syed et al., 2012; Chaudhary et al., 2019b). Estimating the number of AS events is demanding because AS is a dynamic process that changes quickly and depends on the type of the cell or tissue, the developmental stage, and environmental conditions (Chaudhary et al., 2019b). However, recent sequencing technologies allowed us much better to

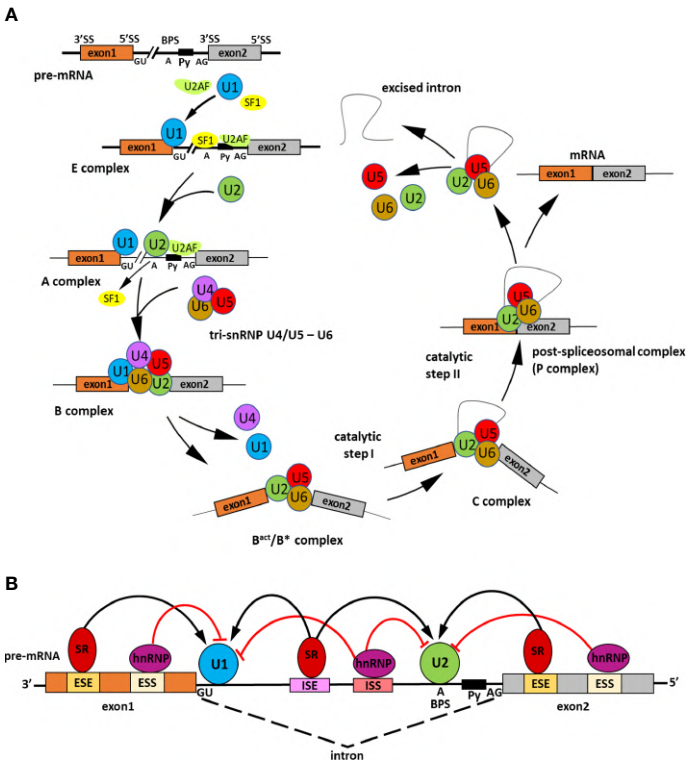


FIGURE 1
Scheme representing the splicing process and its regulation. **(A)** Steps of spliceosome assembly on precursor messenger RNA (pre-mRNA). Precise pre-mRNA splicing requires four constitutive motifs present in the intronic sequence: the 5′ splice site (GU), the 3′ splice site (AG), the branch point sequence (BPS), and the polypyrimidine tract (Py). The intron excision by the spliceosome is divided into several main stages. In the early step of splicing the E complex is generated. Firstly, splicing factor U1 snRNP U1 small nuclear ribonucleoprotein (U1 snRNP) attaches to the 5′ splice site. Next, SPLICING FACTOR 1 (SF1) recognizes the branch point sequence and U2 snRNP auxiliary factor (U2AF) in the polypyrimidine tract. The A complex is created when U2 snRNP displaces SF1. Then, tri-snRNP U4/U5-U6 combines with complex A to form an inactive B complex. The spliceosome is subjected to ATP-dependent structural changes and two U1 and U4 snRNPs detach leading to spliceosome catalytic activation and forming of an active $B^{act}$/B* complex. Next, intron excision is catalyzed in two subsequent transesterification reactions. The first reaction occurs in the C complex and the second in the C*complex. In the C*complex exons are joined together and as a result, is formed a post-spliceosomal complex with ligated exons (P complex). The splicing process is completed when the intron is twisted into a loop and excised; the exons are linked together to form mature mRNA, whereas snRNPs are recycled. **(B)** Role of *cis*-regulatory sequences and *trans*-acting splicing regulators in the spliceosome activity. The *cis*-regulatory sequences include intronic splice enhancers (ISEs) and silencers (ISSs), exonic splice enhancers (ESEs), and silencers (ESSs). To those sequences binds trans-acting RNA binding proteins (RBPs) which promote or inhibit splicing assembly. Serine/arginine (SR) proteins are activators of the splicing that bind ISEs/ESEs and promote exon joining through recruitment of U1 small nuclear ribonucleoprotein (snRNP) to the 5′ splice site and the U2 snRNP to the branch point. On the contrary, the heterogeneous nuclear ribonucleoproteins (hnRNPs) act as splicing inhibitors, attach to ISSs/ESSs, and repress splice site recognition (Gehring and Roignant, 2021).



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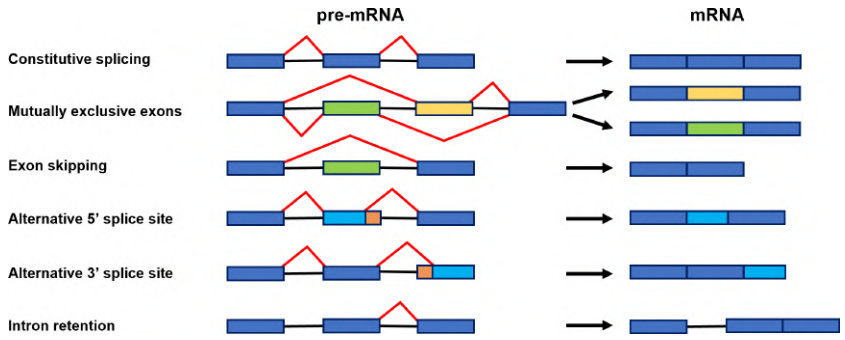


FIGURE 2
Scheme representing constitutive splicing and major alternative splicing events: constitutive splicing, mutually exclusive exons, exon skipping, alternative 5′ splice site, alternative 3′ splice site, and intron retention. Exons are shown as boxes and introns as black lines. Red lines illustrate distinct alternative splicing events (Gurnari et al., 2021).

evaluate genes that are found to undergo AS. It is reported that around 95% of multiexon genes of humans and even up to 83% of plant intron-containing genes are subjected to AS (Pan et al., 2008). Plant genes are shorter than animals' and contain fewer exons and shorter introns. These differences in the structure of genes may be the reason for the different number of AS events in plants and animals (Barbazuk et al., 2008; Chang et al., 2017). The most common event of AS in plants is intron retention. In the case of Arabidopsis, this mechanism accounts for approximately 45%–56% of all AS events. Surprisingly, in plants, only 8% of AS are exon skipping events, in contrast to animals, where this AS event is the most common and represents around 58% of all AS events (Marquez et al., 2012; Reddy et al., 2013; Chaudhary et al., 2019b). Proteins generated due to AS may have altered properties or acquire new biological functions, while losing functional domains results in non-functional proteins (Chaudhary et al., 2019a). The change in the proportion of the functional to non-functional isoforms fine-tunes the developmental processes and allows the plants quickly react to the changing environmental conditions (Kalyna et al., 2012; Ohtani and Wachter, 2019). Alternative splicing also regulates the cell's transcripts level by associating nonsense-mediated mRNA decay (NMD). NMD is the degradation of transcripts containing a premature stop codon (PTC), which causes premature translation termination. The alternatively spliced mRNA isoforms may have a PTC in their nucleotide sequence and then truncated proteins are made from such transcripts. Usually, these proteins are non-functional. Therefore, those aberrant mRNAs are directed and destroyed in the NMD pathway (Drechsel et al., 2013; Nasif et al., 2017). AS has been shown to regulate gene expression through association with the microRNA (miRNA) biogenesis process (Li and Yu, 2021). miRNAs are small and non-coding RNA molecules whose length is around 21–25 nucleotides. They repress the expression of the genes by degrading or inhibiting mRNA translation (Bartel, 2004). AS may occur during primary miRNA transcript (pri-miRNA) processing to mature miRNA. This generates changes in the structure of the pri-miRNA. As a result, the number of functional miRNAs in the cell changes, affecting the expression level of their target genes (Bielewicz et al., 2013; Zhang et al., 2022a). In plants, AS controls the key developmental processes such as growth, circadian clock or flowering time, and adaptation to external conditions, including abiotic stress or pathogen attack (Muhammad et al., 2022). Increasing evidence indicates that AS also regulates seed germination by modulating abscisic acid (ABA) signal transduction (Zhang et al., 2016; Narsai et al., 2017). ABA is the main phytohormone, which through its antagonism with gibberellins (GA), inhibits the process of seed germination (Finch-Savage and Footitt, 2017; Sano and Marion-Poll, 2021). At the seed germination stage, the relevant genes from the ABA signaling pathway, transcription factors, and spliceosome components are alternatively spliced (Table 1).

## The alternative splicing modulates ABA signaling components during seed germination

HYPERSENSITIVE TO ABA1 (HAB1) phosphatase is one of the better described components of the ABA signaling pathway (Saez et al., 2004; Fujii et al., 2009). HAB1 binds and dephosphorylates SNF1-RELATED PROTEIN KINASE 2 (SnRK2) kinases, especially SnRK2.6 (OPEN STOMATA 1 (OST1), and inhibits the transduction of ABA signal (Umezawa et al., 2009; Vlad et al., 2009). The HAB1 belongs to the PP2C phosphatase family, but only in the case of *HAB1* the role of its AS isoforms at the germination stage is known (Wang et al., 2015). As a result of AS of *HAB1*, two protein isoforms are formed that differ in length. The *HAB1.1* is four-exonic, and its translation produces a full-length protein. The second isoform, *HAB1.2* is created due to intron retention between the third and fourth exon, and this intron sequence contains PTC. This variant produces a truncated protein with a partial catalytic domain because of lacking 105 amino acids at the C-terminal end. This defection changes the phosphatase activity of these two AS variants of *HAB1*. The *HAB1.1* and *HAB1.2* can bind to SnRK2, but only the long isoform *HAB1.1* can inactivate it by dephosphorylation, which prevents further ABA signal transduction. Notably, the expression level of both *HAB1* variants is high in seeds, but what is important is that the regulation of seed



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TABLE 1 List of ABA-related alternatively spliced genes that regulate seed germination.

| Gene name (ID gene) | AS isoforms | Description | Reference |
|---|---|---|---|
| *HAB1* (At1g72770) | *HAB1.2* *HAB1.1* | *HAB1.1* positively and *HAB1.2* negatively regulate seed germination. RBM25 is a potential key regulator of alternative splicing of *HAB1*. ACINUS directly interacts with *HAB1 in vivo* and is involved in its splicing. | Wang et al., 2015 Bi et al., 2021 |
| *ABH1*(*CBP80*) (At2g13540) | *ABH1.1* *ABH1.2* | Alternative splicing of *ABH1* is directly mediated by ACINUS. | Bi et al., 2021 |
| *ABI3* (At3g24650) | *ABI3-α* *ABI3-β* | *ABI3* splicing during seed germination is regulated by DRT111 and SUA that act in the same pathway. *ABI3* homologous in wheat, rice, pea, tomato, flax and alfalfa undergo alternative splicing resulting in isoforms with changed functions. | Sugliani et al., 2010; Punzo et al., 2020 McKibbin et al., 2002; Wilkinson et al., 2005; Fan et al., 2007; Gagete et al., 2009; Gao et al., 2013; Wang et al., 2018; Lalanne et al., 2021 |
| ABI5 (At2g36270) | *AT2G36270.1* *AT2G36270.2* | AS splicing of ABI5 is controlled by the splicing factor SKIP. *OsABI5* generates *OsABI5-1* and *OsABI5-2* isoforms with a different ability of binding with OsABI3. | Zhang et al., 2022b Zou et al., 2007 |
| *PIF6* (At3g62090) | *PIF6-α PIF6-β* | *PIF6* is alternatively spliced in seeds and only *PIF6-β* controls germination potential. | Penfield et al., 2010 |
| *PTB1* (At3g01150) *PTB2* (At5g53180) | *SPI* *SPII* | PTBs auto-regulate and cross-regulate their alternative splicing. PTB1 and PTB2 regulate alternative splicing of *PIF6* to control the germination of seeds. | Stauffer et al., 2010 Rühl et al., 2012 |
| *DOG1* (At5g45830) | *DOG1-α* *DOG1-β* *DOG1-γ DOG1-δ* *DOG1-ϵ* | DOG1 enhances ABA signaling through its interaction and inhibition of AHG1 and AHG3. | Née et al., 2017 |
| *SR45* (At1g16610) | *SR45.1 SR45.2* | SR45 regulates the signaling of glucose and ABA at early seedling growth. *SR45* displayed isoform diversity during seed germination. SR45 plays role in salt stress tolerance as a positive regulator. *SR45.1* isoform is essential for salt stress adaptation. | Carvalho et al., 2010 Narsai et al., 2017 Albaqami et al., 2019 |
| *SR45a* (At1g07350) | *SR45a-1a* *SR45a-1b* | SR45a-1a and SR45a-1b negatively regulate response to salt stress at the germination stage. | Li et al., 2021 |

germination in the presence of ABA depends on the *HAB1.2*/*HAB1.1* isoform ratio. A decrease in the level of *HAB1.1* transcripts and an increase in *HAB1.2* turn on the ABA signal transduction. On the contrary, an increase in *HAB1.1* and a reduction in *HAB1.2* turn off the ABA pathway. Studies during seed germination in the presence of ABA using Arabidopsis mutants revealed the role played by *HAB1* isoforms in this process. The *hab1-1* insertional mutant is hypersensitive to ABA. However, the overexpression of *HAB1.1* in the *hab1-1* background leads to an ABA-insensitive phenotype, and transgenic seeds can germinate, in contrast to *hab1-1*. On the other hand, overexpression of *HAB1.2* in the *hab1-1* background does not complement the germination phenotype to the level of WT. All this indicates that those two *HAB1* variants generated in the AS process act antagonistically during seed germination. The role of *HAB1.1* is to promote germination, whereas the role of *HAB1.2* is to inhibit it (Wang et al., 2015). Studies with Arabidopsis mutants in the genes that encode the splicing factors RNA binding motif protein (RMB25), small nuclear ribonucleoprotein E (SmEb), SNW/Ski-interacting protein (SKIP), and ACINUS revealed their involvement in the regulation of AS *HAB1* (Cheng et al., 2017; Bi et al., 2021; Hong et al., 2021; Zhang et al., 2022b) (Figure 3). Mutants in these genes are hypersensitive to ABA during seed germination and show an altered expression pattern of *HAB1.1* and *HAB1.2* isoforms. The RNA binding motif protein (RMB25) contains PWI and RRM motifs important in RNA metabolism and splicing (Zhan et al., 2015). RMB25 interacts with other components of the splicing machinery and controls proper exon junctions (Carlson et al., 2017). ABA activates *RBM25* gene expression, and its translation product, the RBM25 protein, attaches to the last intron in the pre-mRNA of *HAB1* and generates two *HAB1* isoforms, *HAB1.1* and *HAB1.2*. The loss of function of RBM25 increases the *HAB1.2*/*HAB1.1* isoform ratio in the *rbm25* mutant compared to WT (Wang et al., 2015; Cheng et al., 2017). The SmEb protein is one of the seven Sm core proteins (SmB, SmD3, SmE, SmF, SmG, and SmD1) that are part of the pre-mRNA assembly machinery (Reddy, 2007; Schwer et al., 2016). SmEb contributes to regulating ABA signaling during seed germination through alternative splicing of HAB1 and activating ABA-positive regulators such as the transcription factors ABI3, ABI4, and ABI5 (Hong et al., 2021). Studies using *smeb-1*



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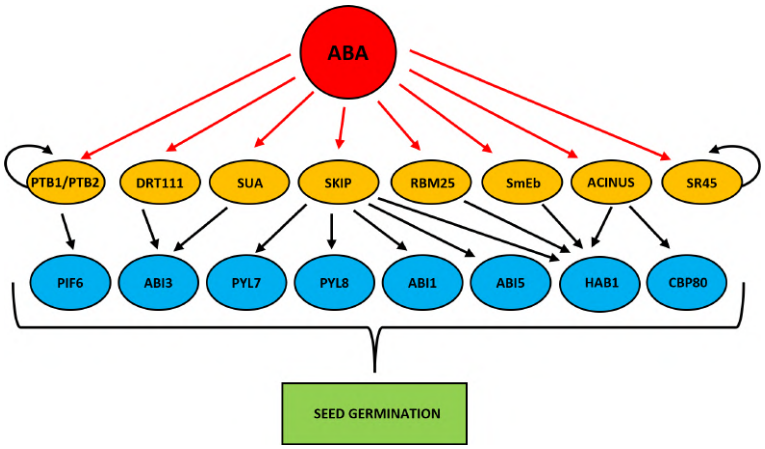


FIGURE 3
Scheme showing ABA-dependent splice factors that regulate alternative splicing of genes involved in the regulation of seed germination. Red arrows indicate ABA activation. Black arrows represent alternative splicing promotion. Curved arrows indicate autoregulation of alternative splicing.

mutants have identified five *HAB1* gene transcripts (*HAB1.1*, *HAB1.2*, *HAB1.3*, *HAB1.4*, *HAB1.5*) from which only two versions of this protein are produced (Hong et al., 2021). Full-length HAB1.1 proteins are produced from *HAB1.1*, *HAB1.3*, and *HAB1.4* transcripts. In contrast, the truncated HAB1.2 protein is formed from *HAB1.2* and *HAB1.5*. After ABA treatment, the transcript levels of *HAB1.1*, *HAB1.3*, *HAB1.4*, and *HAB1.5* decreased in *smeb-1* mutants, but *HAB1.2* increased, resulting in decreased accumulation of the HAB1.1 protein isoform and increased accumulation of HAB1.2 protein. Additionally, only the overexpression of *HAB1.1* in the *smeb-1* background reversed the hypersensitive phenotype of the *smeb-1* mutant to ABA, while overexpression of *HAB1.2* did not restore the WT phenotype. This confirms that the AS *HAB1.1* variant is the functional HAB1 isoform regulating seed germination. Other studies using a mutant in the *SKIP* gene showed that apart from regulating *HAB1* splicing, it also directly regulates AS of other genes of the core ABA signaling pathway, such as PYRABACTIN RESISTANCE-LIKE 7 (PYL7), PYRABACTIN RESISTANCE-LIKE 8 (PYL8), ABA INSENSITIVE 1 (ABI1), and ABI5 (Zhang et al., 2022b). In response to ABA in the *skip-1* mutant, the number of transcripts of functional isoforms of positive ABA regulators PYL7, PYL8, PYL10, ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 1 (ABF1), ABF2, and ABI5 are reduced. On the other hand, the level of incorrect intron retention splicing form in negative regulators such as ABI1 and HAB1 is increased. Significantly, SKIP has previously been shown to interact with the SERINE/ARGININE-rich 45 (SR45) spliceosomal protein and control the circadian cycle through the AS of circadian clock genes (Wang et al., 2012). SKIP also controls the flowering time of plants through the AS and is responsible for plant tolerance to salt stress (Feng et al., 2015; Cui et al., 2017). Recently, the homolog of the mammalian apoptotic inducer of nuclear chromatin condensation (ACINUS) was identified in Arabidopsis (Bi et al., 2021). ACINUS, together with the serine-rich domain RNA-binding protein 1 (RNPS1) and the Sin3A-related protein 18 (SAP18), forms a complex of proteins associated with apoptosis and splicing (ASAP) (Schwerk et al., 2003). ACINUS contains an RNA recognition motif (RRM) required for RNA binding. A similar motif present in ACINUS in Arabidopsis has been identified in its distant paralog, another splicing factor called PININ (PNN). Through this motif, PININ also binds to RNPS1 and SAP18 and forms an alternative PSAP complex (Murachelli et al., 2012). Moreover, these two splice factors are also components of the exon junction complex (EJC), which is deposited onto pre-mRNAs to regulate the export of mRNA, translation, and NMD process (Tange et al., 2005; Schlautmann and Gehring, 2020). Research in Arabidopsis showed that ACINUS and PININ are redundant negative regulators of ABA responses. Knock-out of both *ACINUS* and *PININ* results in an abnormal plant phenotype. Double mutant *acinus-2 pinin-1* is dwarf and exhibits higher ABA sensitivity and delayed germination and flowering time dynamics (Bi et al., 2021). Transcriptomic analysis of the *acinus-2 pinin-1* double mutant exhibited ACINUS and PININ function in the AS mainly by promoting IR events. This type of AS was identified in *HAB1* pre-mRNA. In the mutant seedlings, non-functional *HAB1.2* was over-accumulated, while the short *HAB1.1* isoform was downregulated (Bi et al., 2021). Moreover, not spliced out intron has also been identified in another ABA negative regulator, the *ABA HYPERSENSITIVE 1/CAP BINDING PROTEIN 80 (ABH1/CBP80)* gene. ABH1/CBP80 and CBP20 are creating the CBC complex (Hugouvieux et al., 2001). Mutations in these genes confer ABA hypersensitive phenotype during seed germination (Hugouvieux et al., 2001; Hugouvieux et al., 2002; Papp et al., 2004; Jäger et al., 2011; Daszkowska-Golec et al., 2013; Daszkowska-Golec et al., 2017). The CBC complex co-transcriptionally interacts with the 5′-end cap structure of pre-mRNA and plays the role of an important splicing regulator (Laubinger et al., 2008; Raczynska et al., 2010). In plants, it has been shown that CBC together with the SERRATE (SE) protein promotes the interaction between the U1 small nuclear ribonucleoprotein (U1 snRNA) factor with the donor splice site of the first intron in the pre-mRNA (Laubinger et al., 2008). Studies with the use of single *cbp20* and *cbp80(abh1)* mutants as well as *cbp20/80* double mutant in Arabidopsis showed that a mutation in the *CBP80* gene generates more AS events than



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mutation in *CBP20*, which suggests that larger subunit CBP80 may play a decisive role in AS (Raczynska et al., 2010). In the *acinus-2 pinin-1* double mutant, the intron-containing isoform of *ABH1.2*, which produces a non-functional protein, was significantly accumulated, while the splicing form of *ABH1.1* was decreased (Bi et al., 2021). Together, all these studies indicate that the observed ABA-hypersensitive phenotype of the mutants in the *RMB25*, *SmEb*, *SKIP*, and *ACINUS* may relate to the accumulation of defective isoforms of ABA-negative regulators such as HAB1. Thus, these splice factors turn on or off the ABA signal transduction to fine-tune the seed germination by regulating the functional AS isoforms level in the cell.

## Transcription factors that regulate seed germination undergo ABA-related alternative splicing

AS regulates gene expression by producing various TF isoforms with altered or switched-off DNA binding abilities. This can influence the transcription activation of specific genes and change the transcription rate. One of the better known TFs that undergo AS in plants is ABSCISIC ACID INSENSITIVE 3 (ABI3)/VIVIPAROUS 1 (VP1). ABI3 plays a significant role in the maturation and germination of the seeds and positively modulates ABA signaling by activating ABA-responsive genes (Lopez-Molina et al., 2002; Finkelstein et al., 2008). Expression of *ABI3* is seed-specific and induced by ABA (Parcy et al., 1994; Liu et al., 2013). In Arabidopsis, seeds of *abi3* mutant are insensitive to ABA during germination and highly germinate, indicating that ABI3 negatively regulates seed germination in the presence of ABA (Finkelstein et al., 2008). ABI3 induces the expression of *MIR159*, which encodes the small non-coding RNA miR159. miR159 can regulate ABA signal transduction by targeting two transcription factors MYB DOMAIN PROTEIN 33 (MYB33) and MYB DOMAIN PROTEIN 101 (MYB101), which act as positive regulators of ABA signaling (Reyes and Chua, 2007). Moreover, ABI3 regulates the expression of *ABI4* and *ABI5*, which results in transcription activation of *LATE EMBRYOGENESIS ABUNDANT 1* (*EM1*) and *LATE EMBRYOGENESIS ABUNDANT 6* (*EM6*) genes and leads the inhibition of seed germination (Söderman et al., 2000; Lopez-Molina et al., 2002). Recently, it has been shown that ABI3 can also promote seed dormancy by inducing ABA biosynthesis (Liu et al., 2020). ABI3 binds to the promoter of the *REVERSAL of RDO5 1* (*ODR1*), which is a regulator of ABA biosynthesis, and inhibits its expression. This disrupts the interaction of the bHLH57 transcription factor with ODR1 and as a result, allows bHLH57 to activate the transcription of genes from the ABA biosynthetic pathway, such as *9-CIS-EPOXYCAROTENOID DIOXYGENASE 6* (*NCED6*) and *9-CIS-EPOXYCAROTENOID DIOXYGENASE 9* (*NCED9)* (Liu et al., 2020). However, as well alternative splicing of *ABI3* may affect the seed germination process by altering the function of this protein. AS of *ABI3* was detected in monocots and dicot plants (McKibbin et al., 2002; Fan et al., 2007; Gagete et al., 2009). ABI3 has four conserved and functional domains in its structure (A1 and B1, B2, B3) (Bies-Etheve et al., 1999; Kurup et al., 2000). The acidic A1 domain is located at the N-terminus and activates gene transcription (McCarty et al., 1991). The B1 domain is necessary for ABI3 interaction with other basic leucine zipper (bZIP) family transcription factors, like ABI5, bZIP10, bZIP25, and TRAB1 (Hobo et al., 1999; Ezcurra et al., 2000; Nakamura et al., 2001; Lara et al., 2003 Llorca et al., 2014). In the B2 domain, there is a nuclear localization signal. This domain is also required to transactivate genes containing ABA-responsive elements (ABRE) or G-box motifs (Ezcurra et al., 2000). The B3 domain has DNA binding activity and binds an RY motif (CATGCA), specific for the promoters of genes expressed in the seeds (Suzuki et al., 1997). For the first time, AS of *ABI3* was examined in pea (*Pisum sativum*) (Gagete et al., 2009). In this research were found seven transcripts of *PsABI3* the expression of which was detected during seed development, with the highest level mainly in the middle part of the embryogenesis process. The full-length PsABI3 protein containing all functional domains is generated from the *PsABI3-1* transcript. Functional PsABI3 protein is also produced from the *PsABI3-3* variant, which lacks a fragment located between the A1 and A2 acidic domains. This region contains many amino acids, i.e., proline, serine, and threonine, but its loss does not affect the activity of PsABI3. The PsABI3-1 and PsABI3-3 isoforms can interact with ABI5 due to the presence of the B1 domain. In the case of the *PsABI3-2* transcript, the A2 domain, the region between the two acidic domains, and a fragment of the B1 domain are missing, which generates an inactive protein incapable of interacting with ABI5. However, the presence of the B3 domain in PsABI3-2 may cause the ability of this protein to compete with active PsABI3-1 and PsABI3-3 in binding to promoters of target genes and thus prevent transcription of these genes. The PsABI3-5 protein is active despite having only the A1 domain but with an added short amino acid sequence. Notably, the presence of the A1 domain is insufficient for the biological activity of PsABI3. For this reason, the additional amino acid sequence is likely to cause the activity of the PsABI3-5 protein. The remaining three *PsABI3-4*, *PsABI3-6*, and *PsABI3-7* transcripts produce truncated proteins because of containing a premature termination codon (PTC). Those proteins cannot bind to the promoter sequence of target genes due to the loss of the B3 domain (Gagete et al., 2009). In the model plant Arabidopsis, two *ABI3* transcripts have been identified: *ABI3-α* and *ABI3-β*. The pre-mRNA of *ABI3-α* contains a cryptic intron which in translation produces a full-length protein with four functional domains, A1, B1, B2, and B3, which are important for DNA binding. The lack of a cryptic intron in the pre-mRNA of *ABI3-β* causes the translated protein not to have the B2 and B3 domains and therefore is defective. Analysis of the expression pattern of these AS isoforms revealed that both are expressed in seeds during their development. An increased level of *ABI3-α* transcript is observed during the entire seed development process. In contrast, an increase in the expression of the *ABI3-β* isoform is visible in the final stage of seed maturation (Sugliani et al., 2010). In Arabidopsis, it is known that alternative splicing of *ABI3* is regulated by the conserved splicing factor SUPPRESSOR OF ABI3-5 (SUA) (Sugliani et al., 2010). SUA is a homolog of the human RNA BINDING MOTIF PROTEIN 5 (RBM5), which binds



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to the splicing factor U2AF 65 and is responsible for 3′ splice site recognition (Bonnal et al., 2008). The same in Arabidopsis, SUA also interacts with the U2AF 65 subunit and, inhibiting excising of the cryptic intron, promotes the accumulation of the functional ABI3-α isoform. However, the *sua-1* mutant has a relatively high level of *ABI3-α* transcripts indicating that other splicing factors can replace the function of the SUA in AS *ABI3* during seed maturation (Sugliani et al., 2010). In the case of tomato (*Solanum lycopersicum*), the functional ABI3 protein is formed from the *S1ABI3-F* pre-mRNA (Gao et al., 2013). However, the absence of 90 bp in the pre-mRNA near the region encoding the functional B1 domain results in the formation of a truncated SlABI3-T protein. Interestingly, although *S1ABI3-T* has all four conserved ABI3 domains, its functioning is disturbed, suggesting the region's important role near the B1 domain for the interaction with ABI5. Only the functional SlABI3-F isoform overexpression in tomato seeds causes hypersensitivity to ABA. This may be caused by the shortening of the *SlABI3-T* isoform close to the functional B1 domain, which results in a lower binding ability with ABI5 and consequently affects the efficiency of transcription of the ABI5 target genes and disrupts the ABA signaling. Both *S1ABI3* transcripts are upregulated mainly during seed imbibition, with the expression of the *S1ABI3-F* isoform significantly predominant at the germination stage. However, the use of various phytohormones, as well as abiotic stresses, changed the accumulation ratio of the shortened and full-length isoforms. Also, the expression of seed-specific ABA-dependent genes *SOMNUS* (*SlSOM*), *LATE EMBRYOGENESIS ABUNDANT 1* (*SlEM1*), and *LATE EMBRYOGENESIS ABUNDANT 6* (*SlEM6*) is regulated differently by both isoforms. The S1ABI3-F increases and S1ABI3-T reduces their expression. Interestingly, studies using transgenic plants with ectopic overexpression of *S1ABI3* isoforms in leaves have shown that, in this case, both isoforms function similarly and induce the expression of these ABA-dependent seed specific genes in the leaves. It indicates that the function of S1ABI3-F and S1ABI3-T depends on the tissue in which they are expressed (Gao et al., 2013). The AS of *ABI3* has also been shown in flax (*Linum usitatissimum*) (Wang et al., 2018). In this plant, *ABI3* produces three transcripts *LuABI3-1*, *LuABI3-2*, *LuABI3-3*. Protein containing all functional domains is produced from those transcripts in the translation process. However, only *LuABI3-1* and *LuABI3-2* generate functional proteins. The intron retention in the *LuABI3-3* pre-mRNA causes the generated protein not to exhibit biological activity; like the research under *SlABI3* isoforms in tomatoes and in flax transgenic plants overexpressing *LuABI3-1* or *LuABI3-2* affects the expression of seed-specific genes and thus regulates seed germination (Wang et al., 2018). The latest research on the three stages of seed development of alfalfa (*Medicago truncatula*) showed the presence of three *ABI3* splice isoforms (Lalanne et al., 2021). The first is the full-length MtABI3 protein named SPLICING FORM 1 (SF1), the transcript of which contains nine exons and all functional domains. The *SF2* isoform transcript is truncated, contains eight exons, and lacks the A1 activation domain. The pre-mRNA of the third isoform *SF3* contains six exons, the A1 domain, and truncated B1 and B3 domains but also lacks the B2 domain. The loss of functional domains in two isoforms caused both to have different cell functions. Moreover, each of these isoforms had a different, specific expression pattern during various phases of seed development and a different and specific pattern of expression depending on the type of tested tissue, i.e., embryo, endosperm, and seed coat. Analysis of the differential expressed genes (DEG) in hairy roots after the ectopic expression of individual splicing forms *SF1*, *SF2*, and *SF3* in the background of the *abi3* mutant revealed that only 41 genes are expressed commonly for all three *MtABI3* isoforms. However, 791 specific DEGs for *SF1*, 357 for *SF2*, and 177 for *SF3* were identified as involved in different biological processes. Specific DEGs in *SF1* were associated with photosynthesis, important storage proteins, the photosynthetic PSII complex, and the development of important storage proteins during the seed development process. In the case of *SF2*, the identified DEGs were associated with the plasma membrane intrinsic protein (PIP) and the degradation of fatty acids and the cell wall. On the other hand, the processes involving differential expressed genes specific to the third *SF3* isoform concerned the modification of the cell wall and the activity of pectin methyltransferases. Moreover, alternative splicing of *ABSCISIC ACID INSENSITIVE3/VIVIPAROUS1* (*ABI3/VP1*) homologs has also been reported in monocots such as wheat (*Triticum aestivum*) and rice (*Oryza sativa*) (McKibbin et al., 2002; Wilkinson et al., 2005; Fan et al., 2007). In both species, multiple *ABI3* isoforms have been identified that were generated from multiple different AS events, which usually resulted in an open reading frame (ORF) shift and generated a stop codon. Non-functional shortened proteins from aberrantly spliced transcripts are created, which is usually associated with lower seed quality. As a result, the seeds of these crops show reduced primary dormancy as increased pre-harvest sprouting (PHS).

In Arabidopsis, it is known that ABI3 directly interacts with transcription factor ABI5 *via* the B1 domain and promotes transcription of its target gene by binding to ABRE elements in the DNA (Nakamura et al., 2001). The *ABI5* homolog of Arabidopsis in rice *OsABI5* is alternatively spliced at the seed germination stage generating two variants of transcripts, the *OsABI5-1* and *OsABI5-2* (Zou et al., 2007). As a result of the exon skipping the event in the pre-mRNA *OsABI5*, the translated proteins differ in length in 10 amino acids localized after the bZIP domain. The *OsABI5-2* is an elongated protein version; AS changes the binding activity of *OsABI5* isoforms with ABI3. The ability to bind to ABI3 of both isoforms is preserved. However, the *OsABI5-2* isoform binding is much stronger. This influences the ABI5-ABI3 complex formation efficiency, suggesting different roles of those two *ABI5* isoforms in regulating the ABA signaling under changing environmental conditions (Zou et al., 2007). As previously mentioned, two alternatively spliced isoforms of *ABI5* have also been identified in Arabidopsis in recent studies of the *skip-1* mutant in response to ABA during seed germination (Zhang et al., 2022b).

The PHYTOCHROME INTERACTING FACTORS (PIFs) family consists of the eight proteins belonging to the helix-loop-helix (bHLH) TFs that modulate the response of plants to light and act as negative light regulators in the germination process (Leivar and Quail, 2011; Jeong and Choi, 2013; Leivar and Monte, 2014; Yang et al., 2020). PIF1 and PIF6 directly interact with Phytochrome



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B (PhyB) (Khanna et al., 2004; Narsai et al., 2017). It was revealed that in the presence of light, phytochromes, which are far-red and red-light receptors, are activated and bind to the transcription factor PHYTOCHROME INTERACTING FACTOR 1 (PIF1). This leads to the degradation of PIF1 in ubiquitin and the 26S proteasome pathway and, as a result, enables the seed to germinate (Oh et al., 2004; Shen et al., 2005). In contrast, in the absence of light, the PIF1 protein promotes GA degradation and inhibits the expression of genes from the GA biosynthetic pathway. At the same time, it increases the levels of ABA in seeds by promoting the expression of ABA biosynthesis genes and reducing the expression of genes involved in ABA catabolism (Finkelstein et al., 2008; Oh et al., 2009; Gabriele et al., 2010). In addition to affecting the metabolism of ABA and GA, PIF1 also directly binds to the *ABI3* and *ABI5* genes from the ABA signal transduction pathway and *GA INSENSITIVE DWARF1A* (*GID1A*) and *REPRESSOR OF GA1-3 1* (*RGA1*) genes from the GA signaling pathway (Oh et al., 2007; Oh et al., 2009; Kim et al., 2016a). PIF1 connects the light pathway with the ABA signaling pathway and binds to *ABI3* to activate the expression of the negative seed germination regulator SOMNUS (SOM) to inhibit germination of the seeds (Dong et al., 2008; Kim et al., 2008; Park et al., 2011). Regarding the PIF6 transcription factor, it has been shown that it undergoes AS during seed germination. The expression of PIF6 transcripts is particularly observed in dry seeds, and at the initial stage of germination, there is a strong reduction in its expression level. This expression pattern of PIF6 is contrasted with the PIF1, whose transcripts accumulate only at the imbibition stage (Penfield et al., 2010). As a result of the third exon skipping the event, two *PIF6* transcripts are generated, encoding two different proteins, PIF6-α and PIF6-β. The PIF6-α variant is a full-length protein, while the PIF6-β isoform is truncated and lost the helix-loop-helix bHLH domain essential for binding this transcription factor with DNA. Only seeds overexpressing PIF6-β germinate more intensively compared to the germination level of WT, while PIF6-α overexpression does not affect the germination ratio. This shows that AS caused those two *PIF6* isoforms to affect the seed germination rate differently. It indicates the antagonistic role of *PIF6-α* and *PIF6-β* in the germination process. It is worth mentioning that those two isoforms of *PIF6* do not always act differently. Overexpression of *PIF6-α* or *PIF6-β* inhibited the elongation of the seedling hypocotyl in R-light, which suggests a similar functioning of these isoforms in seedling development (Penfield et al., 2010). It was revealed that AS pre-mRNA *PIF6* is driven by the polypyrimidine tract-binding proteins (PTB), which are the component of the splicosome machinery. PTBs belong to heterogeneous nuclear ribonucleoproteins I (hnRNP I) and act as splicing suppressors (Sawicka et al., 2008; Dai et al., 2022). Arabidopsis genome has three *PTB* genes (*PTB1*, *PTB2*, and *PTB3*) and all of them undergo alternate splicing to generate two transcripts *SPLICING VARIANT I* (*SPI*) and *SPLICING VARIANT II* (*SPII)* (Stauffer et al., 2010). The pre-mRNA of *SPI* is formed into a protein with the correct length, while the *SPII* has PTC in its nucleotide sequence, which directs this transcript to the NMD pathway. This example shows the role of AS coupling with the NMD mechanism in regulating the level of functional proteins. PTB variants can mutually regulate their splicing but also show the autoregulation ability to balance AS events. Studies in Arabidopsis have shown that AS of *PIF6* is regulated only by PTB1 and PTB2 (Rühl et al., 2012). An opposite pattern of accumulation of *PIF6* splice isoforms was observed in mutants overexpressing or with knockdown of *PTB1* or *PTB2*. In the case of mutants with *PTB1* or *PTB2* overexpression and simultaneous overexpression of both *PTB*s, it led to the accumulation of the correct *PIF6-α* isoform. In contrast, the knockdown of single *PTB1* or *PTB2* or both *PTB*s promoted the truncated *PIF6-β* isoform. This contrary accumulation pattern of alternatively spliced *PIF6* isoforms has been associated with the altered germination ability of *PTB* mutants in the presence of ABA. Mutants overexpressing *PTB1* increased the levels of *PIF6-α* and delayed seed germination, suggesting that this protein acts as a negative germination regulator in the presence of ABA. In addition, it was confirmed by the observation that the accumulation of non-functional PIF6-β protein in a double mutant with switched off both *PTB1* and *PTB2* genes increased the seed germination potential in response to ABA. The results of these studies indicate the role of *PTB*s in the AS of *PIF6*. Also, other studies in germinating Arabidopsis seeds identified AS of *PIF6*, in which four differently expressed *PIF6* transcripts were detected (Narsai et al., 2017). Moreover, PhyB, which interacts with PIF6, also undergoes AS (Khanna et al., 2004; Narsai et al., 2017). Two *PhyB* transcripts have been identified that display isoform switch, which suggests that these isoforms may be important at different stages of seed germination. The expression of the first isoform is high in dry seeds and gradually decreases during stratification, while a gradual, high increase in expression is again observed upon exposure to light. In contrast, the expression of the second isoform is the highest during stratification (Narsai et al., 2017). PhyB interacts with the splicing factor DNA-DAMAGE REPAIR/TOLERATION PROTEIN111/SPLICING FACTOR FOR PHYTOCHROME SIGNALING (DRT111/SFPS), which plays a role in photomorphogenesis (Xin et al., 2017). It shows that DRT111 is a hub connecting light and ABA signaling to regulate seed germination (Punzo et al., 2020). The *drt111* mutant was observed to have altered expression of genes from the ABA signaling pathway, including ABI3 and the light signaling pathway e.g. *PIF1* and *PIF6*. Analysis of *drt111-2*/*sua-2* double mutants and *drt111-2/sua-2/abi3-5* triple mutants showed an interaction between *DRT111* and *SUA*. This indicates that DRT111 controls *ABI3* splicing *via* SUA. Variation in the *ABI3* AS isoforms was observed in the single *drt111-2* mutant, where only the non-functional *ABI3-β* isoform was increased in dry seeds. Interestingly, at the imbibition stage, both *ABI3-α* and *ABI3-β* pre-mRNA amounts increased, but ABI3-β levels rise four times more than WT.

## A key regulator of seed dormancy, DOG1 undergoes alternative splicing and interferes with ABA signaling components

Interestingly, the main dormancy regulator DELAY OF GERMINATION 1 (DOG1) has been shown to undergo AS, and



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recent studies have revealed that DOG1 also interacts with the ABA signaling components (Nakabayashi et al., 2015; Dekkers et al., 2016; Née et al., 2017). As a result of the alternative 3′ and 5′ splice sites selection AS events, five different *DOG1* transcripts are generated: *DOG1-α*, *DOG1-β*, *DOG1-γ*, *DOG1-δ*, and *DOG1-ϵ*. These transcripts are translated into three types of proteins because the same protein is produced from the *DOG1-β*, *DOG1-γ*, and *DOG1-ϵ* transcripts (Bentsink et al., 2006; Nakabayashi et al., 2015). Although these proteins vary in length, they are all biologically functional. The role of these splice variants of *DOG1* is unknown. However, current studies suggest they play a synergistic role in seed dormancy (Nakabayashi et al., 2015). Analysis of the phenotype of mutants with overexpression of the individual single *DOG1* isoform in the *dog1* mutant background showed that the seeds do not accumulate the DOG1 protein, resulting in no dormant seeds. However, deep dormancy is observed when the mutant overexpresses at least two *DOG1* isoforms. This phenotype has been associated with the accumulation of the DOG1 protein, which increases with the number of overexpressed isoforms. This shows that at least two *DOG1* isoforms are required to induce seed dormancy. The expression of DOG1 increases with seed development and reaches the highest levels till halfway through the seed maturation process and then decreases until the lowest level in the dry seeds. The rise in the expression of *DOG1* during seed maturation is influenced by low temperature and ABA treatment (Chiang et al., 2011; Kendall et al., 2011). The drop in temperature causes a higher amount of DOG1 protein in the cells, which promotes seed dormancy (Zaretskaya et al., 2022). Analysis of the *dog1-1* mutant revealed its non-dormant phenotype, which is similar to ABA-deficient and signaling mutants. The *dog1-2 cyp707a2-1* double mutant exhibited reduced dormancy compared to the ABA catabolism single mutant *cyp707a2*. The high ABA levels in the *dog1-2 cyp707a2-1* did not lead to seed dormancy (Nakabayashi et al., 2012). However, high levels of *DOG1* in the non-dormant ABA-deficient mutant *aba1* cannot inhibit the germination of the seeds (Bentsink et al., 2006). Interestingly, recent reports indicate that DOG1 enhances ABA signaling through the interaction and inhibition of PP2C ABA HIPERSENSITIVE GERMINATION1 (AHG1) and ABA HYPERSENSITIVE GERMINATION 3 (AHG3) phosphatases characteristic for seed development and germination (Yoshida et al., 2006; Nishimura et al., 2007; Née et al., 2017; Nishimura et al., 2018). Studies using mutants in the *AHG1* and *AHG3*, and *DOG1* genes have shown that the single *ahg1* and *ahg3* mutants are significantly less dormant than the *ahg1/ahg3* double mutant. On the other hand, double mutants *dog1/ahg1* or *dog1/ahg3* can still germinate, and only the loss of the function of all three genes causes seed dormancy. This indicates that AGH1 and AHG3 phosphatases may partially take over their functions and that they are downstream from DOG1 (Née et al., 2017; Nishimura et al., 2018). *AHG3* is regulated in the ABA signaling pathway dependent on PYRABACTIN RESISTANCE/PYR-LIKE PROTEIN/ABA RECEPTOR REGULATORY COMPONENTS (PYR/PYL RCAR) receptors and a separate pathway regulated by DOG1. In contrast, AHG1 phosphatase is regulated exclusively by DOG1).

## Regulation of ABA-related changes in alternative splicing by the spliceosomal machinery during seed germination

Interestingly, it has been shown that spliceosomal components are not only involved in the splicing mechanism but also undergo AS. Serine/arginine-rich (SR) and SR-like proteins belong to the conserved family of pre-mRNA splicing factors. The SR proteins influence the choice of the splicing site and regulate spliceosome assembly by binding to the cis-regulatory sequences in the pre-mRNA (Zahler et al., 1992; Reddy, 2007). In their structure, the SR proteins have one or two RRM motifs at the N-terminus responsible for RNA recognition and an arginine/serine-rich RS domain at the C-terminus allowing for the interaction with other proteins (Wu and Maniatis, 1993; Manley and Tacke, 1996; Graveley et al., 2001; Reddy, 2004; Day et al., 2012). The SR45 protein is an SR-like protein with an additional RS domain located at the N-terminus (Tanabe et al., 2007). In Arabidopsis, *SR45* is spliced at various stages of plant development or in response to external stresses (Ali et al., 2007; Zhang et al., 2014; Filichkin et al., 2015). AS for *SR45*, using alternative 3′ splice sites leads to the production of two isoforms. AS variant *SR45.1* is a full-length transcript, while *SR45.2* is a truncated version. These isoforms encode very similar proteins differing only in eight amino acids, which are only present in the *SR45.1* isoform. Despite this, both isoforms are functional, but surprisingly, each has different functions. The first isoform, *SR45.1*, plays a primary function in flower petal development, while *SR45.2* is in root growth (Ali et al., 2007; Zhang and Mount, 2009; Zhang et al., 2014). Moreover, SR45 has been shown to act in the ABA pathway mediated by glucose (Glc) (Carvalho et al., 2010). Glc induces the expression of ABA biosynthesis and signaling genes, which increases endogenous ABA levels and influences ABA signal transduction (Dekkers et al., 2008; Huang et al., 2016). Such a link between Glc-ABA was observed in the *sr45* mutant, which is hypersensitive to glucose and ABA at the seedling stage (Carvalho et al., 2010). The analysis of the *ABI3* and *ABI5* transcripts levels in the *sr45* mutant after treatment with ABA and glucose showed that these transcripts increased significantly compared to the wild type only after glucose treatment. This indicates that the SR45 protein has a role in the negative regulation of *ABI3* and *ABI5* transcription after glucose treatment. In contrast, reversing the hypersensitivity to Glc of mutant *sr45* in complemented lines with *SR45.1* and *SR45.2* reveals that these isoforms have the same function (Carvalho et al., 2010). Other research studies have shown that the regulatory role of SR45 in the pathway linking sugar signaling with ABA is to control the level of SNF1-RELATED PROTEIN KINASE 1 (SnRK1) in the cell by modulating the AS of INOSITOL POLYPHOSPHATE 5-PHOSPHATASE (5PTase13), which is involved in the proteasomal degradation of SnRK1 (Ananieva et al., 2008; Rodrigues et al., 2013). Analysis of the Arabidopsis seedling transcriptome identified over 4,000 RNAs that directly or indirectly bind to SR45 (Xing et al., 2015). The Gene Ontology (GO) of SR45-related RNA enabled it to indicate the main biological processes in which they are engaged



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and, through this, confirmed the role of SR45 in processes such as flowering, various aspects of plant development, and RNA splicing. Importantly, the highly enriched term GO responded to abiotic stresses and ABA. This showed that SR45 is involved in the ABA signaling pathway. Moreover, the *SR45* knockout affects genes' transcription and the AS from the ABA signaling pathway (Xing et al., 2015). In the case of the *HAB1* gene in the *sr45* mutant, the transcription of this gene was reduced, but the accumulation of the AS spliced variant *HAB1.2* with IR was promoted in the control conditions and in the presence of ABA (Xing et al., 2015). In Arabidopsis, AS isoforms of *SR45* also function in response to salt stress (Albaqami et al., 2019). Knock-out of the *SR45* resulted in increased salt stress sensitivity at different plant life cycle moments, including seed germination. In the presence of salt stress, *sr45* mutant genes related to the *SALT OVERLY SENSITIVE* (*SOS*) pathway and ABA signaling were differentially expressed and/or exhibited changed splicing patterns. Interestingly, only the *SR45.1* long isoform reversed the salt-sensitive phenotype of the *sr45* mutant as well as altered the expression of these genes and splicing patterns. The obtained results indicate that SR45 can positively regulate the response to salt stress in Arabidopsis; in this response, only the longer isoform *SR45.1* is involved (Albaqami et al., 2019). SR45a is another splicing regulator that also undergoes AS and, together with the smaller subunit CAP-BINDING PROTEIN 20 (CBP20) of the CAP-BINDING COMPLEX (CBC), cooperates in the salt stress response (Li et al., 2021). SR45a, similar to SR45, has two RS domains, and because of AS, due to the loss of the C-terminal domain, two isoforms with altered functions are created (Tanabe et al., 2009; Li et al., 2021). SR45a-1a is a full-length functional protein variant, whereas the isoform SR45a-1b is truncated and incapable of interacting with other splicosome elements. However, SR45a-1b plays an important regulatory role. Both SR45a isoforms have the RS domain located at the N-terminal region, so both can interact with the CBP20 protein. In response to salt stress, the complex of SR45a-1a with CBP20 is created, and the binding of those two proteins is enhanced by SR45a-1b. This formed complex regulates the gene expression of the salt-responsive genes and modulates their AS. At the germination stage, the loss of function of *SR45a* confers tolerance to salinity. At the same time, overexpression of the *SR45a-1a* or *SR45a-1b* isoform increased the salinity sensitivity, suggesting a negative regulation of salt stress by the *SR45a* spliced variants (Li et al., 2021). The latest research also indicates the functionality of *SR45* isoforms in seed germination in Arabidopsis. The study of the Arabidopsis transcriptome dynamics during seed germination identified 620 genes that showed isoform variation. Moreover, 612 genes were differentially expressed, including *SR45* (Narsai et al., 2017).

## Transcriptome analyses reveal the involvement of alternative splicing in seeds

Although the amount of RNA-seq research on AS in plants is constantly growing, more data about seeds and the germination process still needs to be collected, especially in monocots. An RNA-seq study of germinating barley (*Hordeum vulgare*) embryos from four varieties identified 2,200 and 3,900 AS transcripts 24 and 48 h after imbibition (Zhang et al., 2016). The alternative 3′ splice site was the most common AS event, which amounted to 34% and 45% of all AS events. This observation was interesting because the most frequent event in plants is intron retention (Reddy et al., 2012). This research showed that AS process changes over time in germinating seeds. The number of detected transcripts after 24 h and 48 h was similar but not identical. Half of the transcripts present in the embryos after 24 h were no longer detectable at 48 h, but other new transcripts were produced at this time. Evaluating the biological functions of these identified AS genes showed their possible engagement in protein synthesis, energy and metabolism of carbon, and post-transcriptional regulation. Moreover, among AS genes, genes acting in the signaling pathways of various phytohormones have been identified. At the 24 and 48 h of germination, splicing variants of genes from the auxin, cytokinin, and the ABA signaling pathway, such as *AUXIN-RESPONSIVE PROTEIN IAA* (*AUS/IAA*) [alternative 3′ splice site (A3S)], *A-TYPE RESPONSE REGULATOR* (*A-ARR*) (IR), or *SnRK2* (A3S, IR), was detected. However, the A3S alternatively spliced variant of the *A-ARR* gene was present only for 24 h and the IR isoform of the *AUX/IAA* gene after 48 h. Exceptionally high levels of coexpressed genes had auxin efflux carrier, auxin-responsive AUX/IAA proteins, and SnRK2 from the ABA signaling pathway. This indicates that this seed germination stage involves phytohormones such as auxin and ABA. Therefore, the newly-created splice variants may differently modulate various signaling pathways and biological processes and thus affect seed germination. AS was also reported in rice seeds in response to stress conditions (Chen et al., 2019). In low oxygen conditions, 1,741 differentially expressed AS were identified. Interestingly, only 5% of AS genes were as well differentially expressed. That result reveals the independent transcription role of alternative splicing in gene regulation. A similar relation was found in the study of the Arabidopsis transcriptome, where only 6% of the identified genes that underwent AS were also differentially expressed (Srinivasan et al., 2016). In 14 and 20 days after pollination, 4,723 and 4,494 AS genes were identified, respectively. Those genes were mainly involved in processes related to mRNA catabolism. In total, 8,927 AS events were detected, of which 88% were newly identified in Arabidopsis. Among them was found a new alternative 3′ splice site (A3P) event of the *FUSCA3* (*FUS3*) gene which codes for a B3 domain transcription factor that acts as a regulator of seed development (Gazzarrini et al., 2004; Tsai and Gazzarrini, 2012). From this AS variant a truncated protein was created due to reading frame shift and formation of a premature stop codon within the B3 domain. This may influence FUS3 functionality and change its DNA binding activity. Although the function of this splice isoform is not determined, it is worth noting that the A3P *FUS3* was expressed explicitly at 20 DAP, in contrast to the full-length isoform which was downregulated. The high frequency of the AS process in seeds was also confirmed by RNA-seq studies in dicots (Aghamirzaie et al., 2013; Ruan et al., 2018; Feng et al., 2019; Liu et al., 2022). In the case of peanut (*Arachis hypogaea*) seeds, 30 days after flowering,



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20,213 genes underwent AS, which is 49.69% of all expressed genes. In contrast, on the 50th day after flowering, 19,534 AS genes were identified, corresponding to 48.30% of all expressed genes. 92,483 and 85,562 AS events were detected at these two-time points, respectively. It is worth noting that in the seeds was the most significant number of AS isoforms compared to the root and leaf tissue. It was observed that a considerable percentage of genes that underwent AS was involved in the metabolism of fatty acids (approx. 60%), which is an important process for the development of oil seeds. This suggests that alternative splicing may be a crucial mechanism regulating this process. Also, the new splice variants of *Aradu.5N10F*, *Araip.84LR8*, and *Araip.92Q2X* genes encoding fatty acid desaturase (FAD) were detected. These isoforms were generated as a result of a novel exon presence. However, in the case of the *Araip.92Q2X*, an additional isoform was also produced which, except for an extra exon, had as well a 3′ alternative exon end (3′-AE). All those newly detected AS events did not disrupt the translation process and resulted in the formation of correct proteins (Ruan et al., 2018). In the soybean (Glycine max) case, 217,371 different AS transcript isoforms from 47,331 AS genes were identified at the stage of embryogenesis (Aghamirzaie et al., 2013). Moreover, RNA-seq studies of germinating seeds showed high AS process dynamics. In cotton (*Gossypium australe*), the greatest differences in AS were observed in comparison of the 5th and 30th hour of germination (Feng et al., 2019). Similarly, RNA-seq studies in Arabidopsis showed variable accumulation of AS isoforms at different phases of seed development and in various tissues, with the most significant changes when comparing seeds after imbibition with dry seeds (Narsai et al., 2017). The AS pattern was recently investigated in seeds of high and low secondary dormancy varieties of oilseed rape (*Brassica napus*) after polyethylene glycol 6000 (PEG6000) treatment to induce secondary dormancy. After treatment with PEG, 5,136 genes that underwent AS were identified, accounting for 8% of all expressed genes. From these genes, 11,408 splicing variants have been detected, of which over 50% were generated due to intron retention. Among the differentially alternatively spliced genes, a large group consisted of genes encoding spliceosome components, e.g., small nuclear ribonucleoprotein molecules (snRNPs), serine/arginine-rich proteins (SR) whose function in Arabidopsis has been confirmed in the process of embryo development (*JANUS*), seed germination (*RZ-1C*, *RBM25*) or response to stress conditions (*SCL30A*, *U1-70K*, *SR45A*). Moreover, within the differentially alternatively spliced genes, 342 genes played a role in the secondary dormancy, with only seven of them also significantly expressed. Interestingly, the expression of genes that underwent AS and were also differentially expressed was more than three times higher than the rest of the differentially expressed genes, indicating a correlation between changes in the AS process with changes at the transcription level. However, comparing the number of differentially alternatively spliced genes with differentially expressed genes showed that the number of unique genes is greater than the number of common genes and that suggests an independent action of AS process and transcription in seed dormancy of oilseed rape (Liu et al., 2022).

The RNA-seq data combined with proteomics analysis complements the information about the functions of generated splicing isoforms and may enrich the knowledge of the mechanisms regulating the seed germination process. This approach was used in the study of maize (*Zea mays*) seed germination in the presence of a high concentration of NaCl. It was noted that during germination, the reaction to the salt stress of the embryo and the endosperm is different. In the salt stress conditions, the embryo could germinate, whereas the germination of seeds with endosperm was inhibited. However, in the presence of ABA, germination was strongly reduced in both cases. These interesting observations suggest that, unlike the endosperm, the embryo is not responsible for the perception of the NaCl, but the signal transduction of the ABA is active. The transcriptomic and proteomics analyses showed significant differences in posttranscriptional events and translational regulation between the embryo and the endosperm. Comparable amounts of differentially expressed posttranscriptional events (DPTE) were detected in the embryo and the endosperm tissue, respectively – 14,570 events of 4,529 genes in the embryo and 15,023 events of 4,756 genes in the endosperm. These events included processes that generate different mRNA isoforms, such as the alternative start of transcription, alternative splicing, and alternative polyadenylation. In contrast, few differentially expressed genes were detected in both examined tissues, just 57 in the embryo and 148 in the endosperm. However, proteomics analysis showed the presence of 243 proteins with increased and 419 proteins with reduced expression in the embryo after NaCl treatment. In the case of endosperm, these amounts amounted to 389 and 234, respectively. Interestingly, among the proteins that were produced as a result of DPTE, 11 splicing factors were identified, and their expression at the protein level was differently regulated in the embryo and endosperm. Together, these studies indicate that posttranscriptional events, including AS, may be crucial for seed germination in response to salt stress and that the different expression patterns of splicing factors can be connected with the opposite reaction to NaCl of the embryo and endosperm in maize (Chen et al., 2021).

## Third-generation sequencing (TGS) in plant transcriptome studies

So far, genome-wide analysis of AS has been primarily based on high-throughput sequencing technology using the RNA-seq Illumina short-read approach. However, detecting various isoforms, repeated sequences, and transposable elements using Illumina is difficult. This is because it is impossible to distinguish different transcripts with identical exons, so reconstructing full-length alternative transcripts is demanding (Bernard et al., 2014). TGS like Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) overcome these limitations due to the use of SMRT sequencing technology which allows the sequencing of single molecules in real time (Zhao et al., 2019). The long-read RNA sequencing technologies can sequence full-length transcripts and obtain long cDNA or RNA reads. This allows for identifying new



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isoforms of transcripts, protein-coding or non-protein–coding genes, and alternative polyadenylation sites. Long reads also remarkably improve the plants' transcriptome annotation (Abdel-Ghany et al., 2016; Zhang et al., 2019; Cui et al., 2020). This together makes PacBio and ONT more often used to analyze plant transcriptomes. TGS has been recently applied in transcriptomic studies of many plant species such as maize (*Zea mays*) (Wang et al., 2016), rice (*Oryza sativa*) (Zhang et al., 2019), sorghum (*Sorghum bicolor*) (Abdel-Ghany et al., 2016), rapeseed (*Brassica napus*) (Yao et al., 2020), strawberry (*Fragaria vesca*) (Li et al., 2018), bamboo (*Phyllostachys edulis*) (Wang et al., 2020), Arabidopsis (Li et al., 2016), and tea plant (*Camellia sinensis*) (Qiao et al., 2019). A maize multi-tissue analysis using PacBio revealed 111,151 transcripts, of which 57% represent a novel, also specific for the tissue isoforms. By discovering new genes, isoforms, and lncRNAs, this method more than doubled the number of alternative transcripts and updated the annotation of the maize genome (Wang et al., 2016). To increase assembly quality and reduce the error rate, hybrid sequencing combining NGS and TGS approaches is applied. In the transcriptome study by PacBio and Illumina of adzuki bean (*Vigna angularis*), two varieties during seed germination under drought stress, 2,457 gene loci, and 46,177 isoforms were newly discovered, and the quality of transcript was significantly improved (Zhu et al., 2020).

## Concluding remarks

Alternative splicing (AS) is a crucial co-transcriptional mechanism that regulates gene expression in response to internal cues and environmental signals. Advances in high-throughput sequencing (HTS) technology showed plants' increasing frequency of AS events. However, there still needs to be more information regarding the factors that regulate AS and the effects of AS on mRNA levels and protein functioning. Considering the link between AS and seed germination, further studies are needed to uncover the complex regulatory networks of seed germination and reveal parts of splice variants. This opens another exciting area for research. The obtained knowledge can be used to regulate gene expression to develop crops with new and desirable functional traits.

## Author contributions

ES wrote the first draft of the manuscript and prepared the figures and table. AD-G contributed to the conception and design of the manuscript and revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Publikacja 2**

**Sybilska E.**, Collin A., Sadat Haddadi B., Mur L.A.J., Beckmann M., Guo W., Simpson C.G., Daszkowska-Golec A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (*Hordeum vulgare*). *Scientific Reports*, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9



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# The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (*Hordeum vulgare*)

Ewa Sybilska[1], Anna Collin[1], Bahareh Sadat Haddadi[2], Luis A. J. Mur[2], Manfred Beckmann[2], Wenbin Guo[3], Craig G. Simpson[4] & Agata Daszkowska-Golec[1]✉

**To decipher the molecular bases governing seed germination, this study presents the pivotal role of the cap-binding complex (CBC), comprising CBP20 and CBP80, in modulating the inhibitory effects of abscisic acid (ABA) in barley. Using both single and double barley mutants in genes encoding the CBC, we revealed that the double mutant *hvcbp20.ab/hvcbp80.b* displays ABA insensitivity, in stark contrast to the hypersensitivity observed in single mutants during germination. Our comprehensive transcriptome and metabolome analysis not only identified significant alterations in gene expression and splicing patterns but also underscored the regulatory nexus among CBC, ABA, and brassinosteroid (BR) signaling pathways.**

**Keywords** ABA, Alternative splicing, Barley, Cap-binding complex, Embryo, Germination, Transcriptome

Seed germination encompasses a variety of processes that occur from imbibition to radicle emergence[1]. Monocot seeds comprise three main components: the embryo, the endosperm, and the protective seed coat[2]. Plant studies have revealed that embryos are transcriptionally and translationally active, and crucial for seed germination and plant establishment[3–9]. Spatial transcriptomic analysis of germinating barley grains showed high expression of genes involved in cell division, lipid metabolism and transfer, phytohormone signaling, and aquaporin in embryos. Additionally, transcription factors and translation-related genes are mainly expressed in embryonic tissues[9]. Interestingly, other studies have reported that more than 12,000 transcripts are stored in dry barley grains and activated during seed germination[4].

Regulation of the seed germination process at the molecular level requires interaction between various phytohormones but demands a critical balance between abscisic acid (ABA) and gibberellic acid (GA)[10–12]. During dormancy regression, ABA degradation and GA biosynthesis is known to increase. High ABA content in seeds prolongs dormancy and delays seed germination. Thus, ABA and GA antagonistically control the seed germination process[13]. Plants have evolved complex mechanisms to adapt to the changing environmental conditions. One of these mechanisms is the inhibition of seed germination under stressful conditions. Various abiotic factors have been shown to initiate cellular ABA accumulation and the activation of ABA signaling[14–18]. The central player in ABA-mediated inhibition of seed germination is the basic leucine zipper (bZIP) transcription factor ABA INSENSITIVE5 (ABI5)[19,20]. In addition to ABA and GA, other phytohormones, including brassinosteroids (BRs), also control the seed germination process[21]. BRs antagonize ABA during seed germination by inhibiting the ABA signal transduction pathway[22]. This aligns with the observation that the BR signaling *bri1* (*brassinosteroid insensitive 1*) and *bin2-1* (*brassinosteroid-insensitive 2*) mutants and the BR biosynthesis *det2-1* (*deetiolated 2*) mutant, are hypersensitive to ABA during seed germination[13,23–26]. The critical repressor of BR signaling, BRASSINOSTEROID INSENSITIVE2 (BIN2), interacts with and phosphorylates SNF1-RELATED PROTEIN

[1]Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Jagiellońska 28, 40-032 Katowice, Poland. [2]Department of Life Science, Aberystwyth University, Aberystwyth, UK. [3]Information and Computational Sciences, James Hutton Institute, Dundee DD2 5DA, Scotland, UK. [4]Cell and Molecular Sciences, James Hutton Institute, Dundee DD2 5DA, Scotland, UK. ✉email: agata.daszkowska@us.edu.pl



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KINASE 2 (SnRK2) to promote its kinase activity[27]. Other studies have shown that, during seed germination, BIN2 stabilizes ABI5 through direct interaction and phosphorylation[22].

Genes encoding crucial components of the ABA signaling pathway, the spliceosome and transcription factors undergo alternative splicing (AS), to generate various splice isoforms that fine-tune the seed germination process[28–33]. In AS multiple mRNA isoforms are produced from a single gene through differential intron and exon excision or retention in various combinations from pre-mRNA. Thus, AS regulates gene expression at the co-transcriptional level and increases protein and transcriptome diversity[34,35].

The *CBP20* (*cap-binding protein 20*) and *CBP80* genes encode the small and large subunits of the 5' cap-binding CBC (cap-binding complex), respectively. CBC is involved in conserved processes related to RNA metabolism, including miRNA biogenesis and alternative splicing[36–38]. CBP20 and CBP80 are RNA-binding proteins (RBPs), and their interaction is essential for complex functionality and binding to RNA[39]. The CBC subunits were initially characterized in human HeLa cells, and subsequent studies identified their homologs in other organisms, including plants[40]. Interestingly, the aminoacid sequences of both CBC subunits are highly conserved across the species, including yeast, animals, and plants, confirming the regulatory importance of the CBC complex[41]. The Arabidopsis AtCBP20 protein shows 68% identity and 82% similarity to human CBP20 and approximately 53% identity and 77% similarity to its ortholog in yeasts. However, the larger AtCBP80 subunit is less conserved, showing 28% identity and 50% similarity to the human ortholog, as well as 22% identity and 42% similarity to the yeast protein[38]. In Arabidopsis, the CBP20 protein consists of a 138-amino acid core (N-terminal) region containing the RNA binding domain (RBD) with RNP2 and RNP1 motifs and a plant-specific 120-amino acid C-terminal tail. The RBD domain plays a crucial role in recognizing and binding mRNA cap structures, while the N-terminal part is essential for interacting with CBP80, stabilizing CBP20. The C-terminal region includes nuclear localization signals (NLS), facilitating the transport of the CBC complex from the cytoplasm to the cell nucleus. Unlike in animals, the plant CBP80 subunit lacks NLS signals and requires CBP20 for nuclear transport[41–43]. Computational modeling of CBP20 in barley revealed the presence of NLS in C-terminal region of protein[43].

It is worth to note that mutants in genes encoding CBC subunits are viable and not very different phenotypically from the WT under optimal conditions. The *cbp20* and *cbp80* single mutants have been investigated in several plant species, including Arabidopsis, barley, and potatoes[43–49]. However, research on the *cbp20/cbp80* double mutant has been limited to molecular studies in mature Arabidopsis plants. This research discovered a profound role of the CBP80 subunit in AS regulation[38].

Our study focused on barley (*Hordeum vulgare)* as an object, a diploid species (n = 7). The barley genome contains a single copy of the *HvCBP20* and *HvCBP80* genes. Single mutations in the *CBP20* and *CBP80* genes cause hypersensitivity to ABA during seed germination and confer drought tolerance to dicots and monocots[36–38,50]. To address the gap in knowledge regarding the function of the cap-binding complex (CBC) in the ABA signaling pathway during barley seed germination, we used TILLING to identify mutants *hvcbp20.ab* and *hvcbp80.b* in both *HvCBP20* and *HvCBP80* genes. We then crossed the single mutants to produce and identify the homozygous double mutant *hvcbp20.ab*/*hvcbp80.b*. We found that mutations in both CBC subunits resulted in a distinct ABA response that differed from that of single mutants. Through transcriptomic analyses, we identified differential gene expression and alternative splicing patterns. Notably, the *hvcbp20.ab*/*hvcbp80.b* double mutant exhibited altered AS regulation and significant changes in brassinosteroid signaling following ABA exposure which facilitated seed germination.

## Results

### Barley mutant in both CBC subunits is insensitive to ABA during seed germination

In assessing ABA sensitivity at 1 DAI (Days After Imbibition) the *hvcbp20.ab*/*hvcbp80.b* (hereafter referred to as the double mutant) showed germination rates comparable to those of the wild-type (WT) when exposed to 75 µM ABA, in contrast to single mutants that exhibited significant germination inhibition (Fig. 1a–c). By 7 DAI, both the double mutant and WT seeds exceeded 90% germination, suggesting that the double mutation may counteract ABA-mediated inhibition, possibly by modulating the ABA-signaling pathway (Fig. 1d).

### Global analysis of *hvcbp20.ab/hvcbp80.b* embryos transcriptome in response to ABA

To better understand how the double mutant responded to ABA during seed germination, we performed a comparative analysis of the transcriptomes of all genotypes in the presence of ABA and under control conditions.

Under control conditions, there were only a few transcriptomic changes in all tested mutants compared to the wild type (WT). However, ABA treatment significantly increased the numbers of DEGs and DETs (Table 1). The double mutant in *CBP20* and *CBP80* genes showed the greatest transcriptional changes in response to ABA compared to the other genotypes. The numbers of differentially alternatively spliced (DAS) genes, differentially expressed transcripts (DETs), and differential transcript usage (DTU) was the highest in the double mutant. This number in *hvcbp20.ab*, *hvcbp80.b* and WT were reduced by over 30% compared to the amount of DAS, DET, DTU identified in the double mutant. Moreover, the double mutation significantly affected alternative splicing and transcript isoform formation. The intersection between DE/DAS genes and DET/DTU showed that 69 genes underwent AS only in the WT, 60 in *hvcbp20.ab*, 62 in *hvcbp80.b*, and 108 in the double mutant, corresponding to 68, 58, 60, and 71% of all DAS genes, respectively (Supplementary Fig. S2a). This suggests that DAS genes are mainly regulated at the alternative splicing level. Conversely, most DTU transcripts were produced by transcription in conjunction with AS, with DTU alone comprising 40% of the double mutant (Supplementary Fig. S2b).


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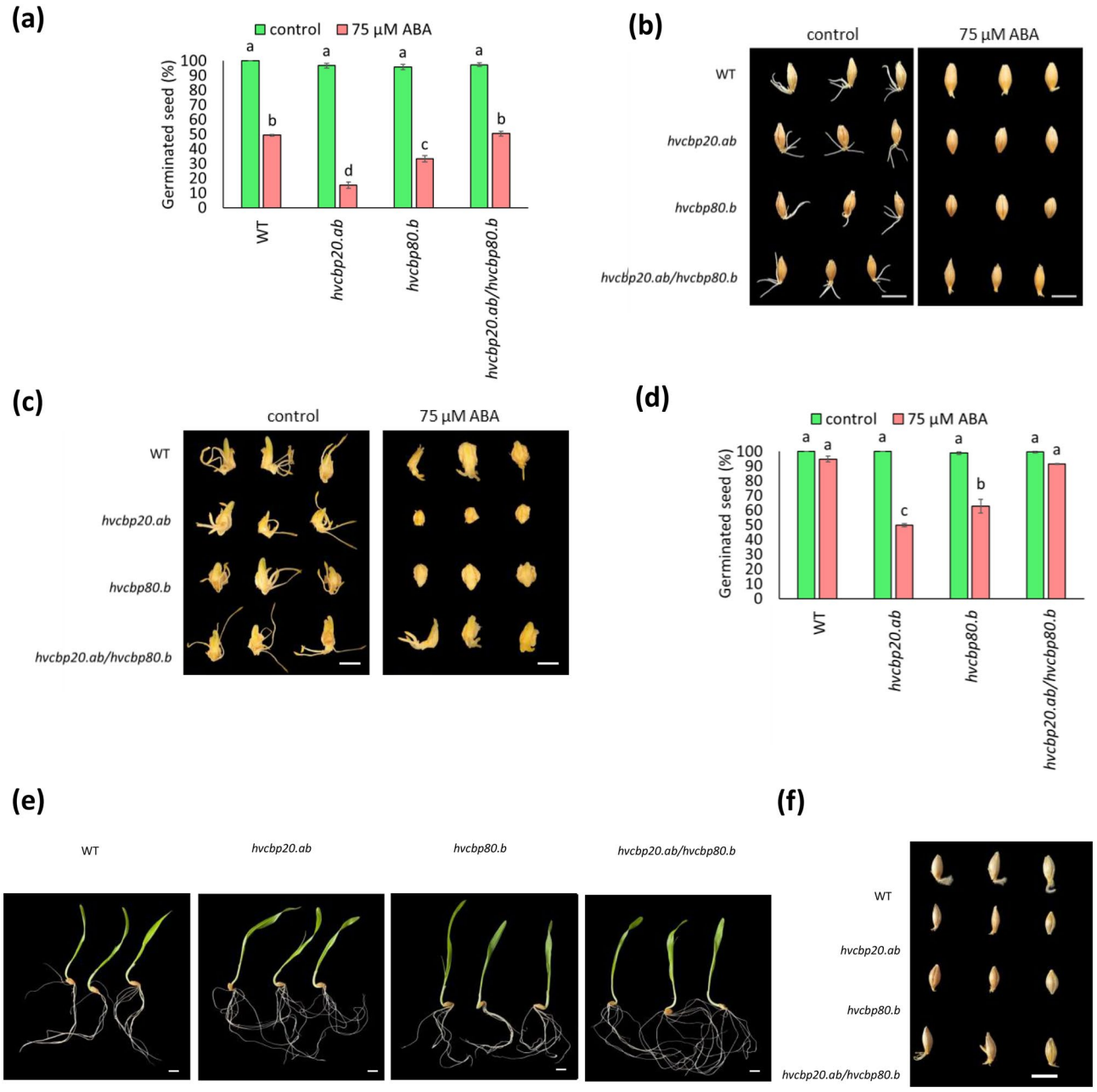


**Figure 1.** Germination of *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b* and the WT in the presence of 75 µM ABA. (**a**) Seed germination percentage at 1 DAI. (**b**) Seed germination phenotypes at 1 DAI. Bar = 1 cm (**c**) Embryo germination phenotypes at 1 DAI. Bar = 3 mm. (**d**) Seed germination percentage at 7 DAI. (**e**) Seed germination phenotypes at 7 DAI in control conditions. (**f**) Seed germination phenotypes at 7 DAI in ABA presence. Bar = 1 cm DAI (day after imbibition). Statistical analyses were performed using one-way ANOVA ($P \leq 0.05$) with post-hoc Tukey HSD (Honestly Significant Difference). Statistically significant differences ($P \leq 0.05$) are marked by different lower-case letters.

## Co-expression analysis reveals clusters of genes with a similar expression pattern in double mutant and WT in the presence of ABA

Co-expression analysis of DEGs (8070) identified in all genotypes tested in response to ABA during the seed germination revealed 12 gene clusters (Fig. 2a; Supplementary Data S1). The C8 and C11 clusters showed consistent expression patterns between the WT and the double mutant, contrasting with *hvcbp20.ab* and *hvcbp80.b*. In the C8 cluster, genes were upregulated in both the double mutant and WT, while being downregulated in the single mutants. On the contrary, the C11 cluster contained genes downregulated in the WT and double mutant but upregulated in *hvcbp20.ab* and *hvcbp80.b* single mutants. The Gene Ontology (GO) enrichment analysis of these clusters identified key biological processes that were prevalent in DEGs with similar expression patterns. In the C8 cluster, the glycolipid catabolic process (GO:0019377), carbohydrate metabolic process (GO:0005975), maintenance of location (GO:0051235), vegetative phase change (GO:0010050) and glutamate catabolic process


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| Contrast | DE genes | DAS genes | DE transcripts | DTU transcripts |
|---|---|---|---|---|
| ABA.WT-control.WT | 5533 | 102 | 8222 | 118 |
| ABA.cbp20-control.cbp20 | 5657 | 103 | 8541 | 131 |
| ABA.cbp80-control.cbp80 | 5914 | 103 | 9424 | 129 |
| ABA.double-control.double | 6309 | 152 | 11,733 | 196 |
| control.cbp20-control.WT | 19 | 2 | 16 | 2 |
| control.cbp80-control.WT | 85 | 5 | 46 | 3 |
| control.double-control.WT | 77 | 4 | 51 | 9 |

**Table 1.** Number of DE/DAS genes and DE/DTU transcripts in different contrast groups. *DE* differential expression, *DAS* differential alternative splicing, *DTU* differentia transcript usage.

(GO:0,006538) were the most significant GO terms (Fig. 2b). Whereas in the C11 cluster the top GO-BP were amino acid transmembrane transport (GO:0003333), organic acid transmembrane transport (GO:1903825), protein palmitoylation (GO:0,018,345), negative regulation of floral organ abscission (GO:0060862) and negative regulation of dephosphorylation (GO:0035305) (Fig. 2c).

## ABA-Induced expression of splicing factors and brassinosteroid signaling regulators unique to double mutant germination

We identified 642 DEGs specific to the double mutant. The single mutants *hvcbp20.ab* and *hvcbp80.b* displayed 241 and 591 unique DEGs, respectively, whereas the WT had 281 DEGs (Fig. 2d). Notably, the gene expression profile of the double mutant was significantly higher, with implications for the distinct from single mutants germination phenotype observed under ABA treatment. GO enrichment analysis of the upregulated gene set in the double mutant showed biological processes (BP) such as nucleic acid phosphodiester bond hydrolysis (GO:0090305)), suggesting an upregulation of splicing activity (Supplementary Data S2, Supplementary Data S3). Notably, six splicing factors have been identified, each with homologs in Arabidopsis, thereby providing a phylogenetic anchor for functional suggestions (Fig. 2e). These include CELL DIVISION CYCLE 5 (CDC5; BaRT2v18chr5HG272090), U2af small subunit A (U2AF35A; BaRT2v18chr5HG254110), SUPPRESSOR OF ABI3-5 (SUA; BaRT2v18chr3HG159600), embryo-defective 2016 (EMB2016; BaRT2v18chr7HG366170), and putative branchpoint-bridging protein-like (SF1; BaRT2v18chr2HG081650) (Supplementary Data S4). Conversely, genes implicated in the negative regulation of brassinosteroid signaling pathways (GO:1900458) were downregulated, highlighting a potential mechanistic intersection between ABA and BRs signaling, REMORIN (REM4.1; BaRT2v18chr2HG069620), BRI1 KINASE INHIBITOR 1 (BKI1; BaRT2v18chr5HG251390), and a previously uncharacterized gene (BaRT2v18chr5HG272750) (Supplementary Data S5). Collectively, these results delineate a distinct transcriptional landscape for the double mutant, with implications for its exclusive ABA-mediated germination phenotype, mediated in part by modulations in the splicing and brassinosteroid signaling pathways.

## Regulatory interplay between transcription factors, splicing factors, and brassinosteroid signaling inhibitors in double mutant

To elucidate the transcription factors (TFs) that potentially orchestrate the regulation of gene expression in response to ABA, we analyzed DEGs after ABA treatment across all genotypes (Supplementary Data S6). This yielded a list of 24 TFs in the double mutant, which starkly contrasts with the 6, 21, and 9 TFs found in *hvcbp20.ab*, *hvcbp80.b*, and WT, respectively. Notably, most TFs identified in the double mutant were upregulated, corroborating the enhanced transcriptional activity observed in these embryos upon ABA exposure.

The TFs encompassed 12 distinct transcription domain families (Supplementary Table 1), suggesting diverse regulatory capacities for modulating ABA responses during the seed germination stage. We next focused on prediction of transcription factor-binding sites (TFBSs) within promoters of DEGs specific to the double mutant. In response to ABA, six TFs were associated with 1178 TFBSs among the 392 DEGs specific to the double mutant suggesting potential regulatory complexity (Supplementary Data S7). A subset of these TFs was identified as having binding sites within DE genes encoding critical splicing factors. One TFBS was within the promoter of the negative regulator of BR signaling (REM4.1; BaRT2v18chr2HG069620) and one TFBS within *serine/threonine-protein kinase SAPK10* (SAPK10; BaRT2v18chr7HG385230) (Supplementary Data S8). These results suggest that the identified TFs may modulate the expression levels of genes relevant to the seed germination phenotype of double mutants in the presence of ABA, which may be CBC-dependent.

## Alternative splicing is impaired in germinating embryos of hvcbp20.ab/hvcbp80.b in the presence of ABA

To compare changes in the alternative splicing pattern in the double mutant, *hvcbp20.ab*, *hvcbp80.b* and the WT in response to ABA, the sets of DAS genes were compared (Fig. 3a). Most genes that underwent splicing were uniquely spliced in each genotype. However, the largest number of DAS genes was identified in the double mutant (Supplementary Data S9). To identify the specific transcripts contributing to the classification of a gene as DAS, we performed a differential transcript usage (DTU) analysis. This process identified individual transcripts that exhibited significant differences relative to the other transcripts from the same gene. In the double mutant, 72% of exclusive DAS genes contained DTU transcripts. For *hvcbp20.ab* and *hvcbp80.b*, the DAS&DTU amounted


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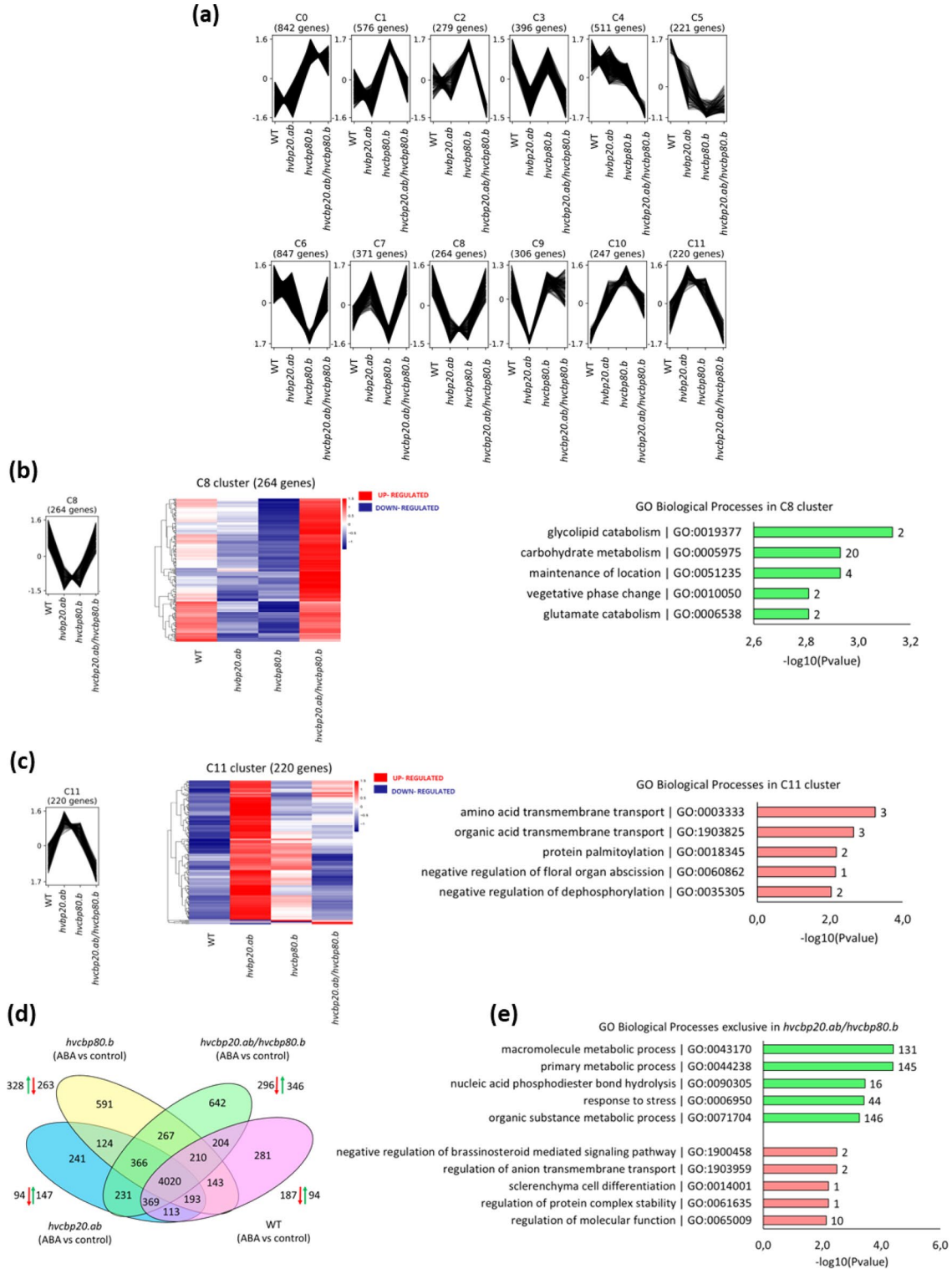


**Figure 2.** Global analysis of differentially expressed genes (DEG) of *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b* and WT after 75 µM ABA treatment compared to control conditions. (**a**) Co-expressed clusters of DEGs in response to ABA. (**b**) Heatmap of DEGs and enriched GO biological processes in the C8 cluster. (**c**) Heatmap of DEGs and enriched GO biological processes in the C11 cluster. (**d**) Venn diagram of DEGs displaying up- and downregulated exclusively expressed genes in each contrast group. (**e**) Overrepresented GO biological processes among upregulated and downregulated exclusive DEGs in *hvcbp20.ab/hvcbp80*.b.


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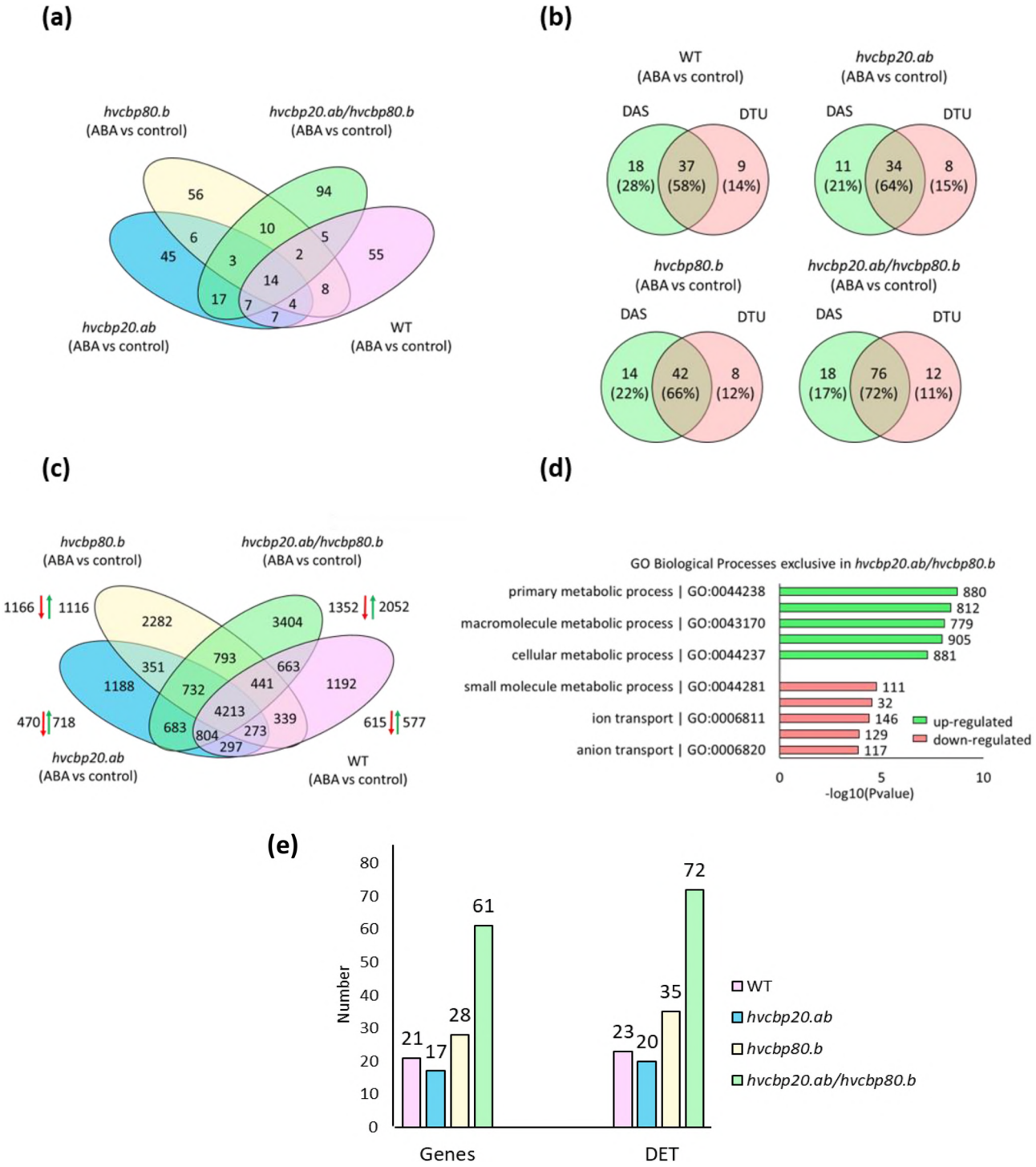


**Figure 3.** Global analysis of genes with differential alternative splicing (DAS) genes and differentially expressed transcripts (DET) in *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b* and WT after 75 µM ABA treatment compared to control conditions. (**a**) Venn diagram of DAS genes in each contrast group. (**b**) Venn diagrams displaying the amount of exclusive DAS genes with DTU transcripts. (**c**) Venn diagram of DETs displaying up- and downregulated exclusively expressed transcripts in each genotype. (**d**) Overrepresented GO biological processes among upregulated and downregulated exclusive DETs in *hvcbp20.ab/hvcbp80.b*. (**e**) The number of genes encoding transcripts of splicing factors (SF) with the corresponding number of transcripts of SFs among exclusive DET in each genotype.

to 64% and 66%, respectively. In contrast, the lowest number (58%) of exclusive DAS genes with corresponding exclusive DTU was identified in the WT (Fig. 3b; Supplementary Data S10). Hence, these data indicate that the


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double mutation exerts a pronounced effect on AS, resulting in a significant number of unique DTUs that align with the elevated number of DAS genes.

## Transcripts of splicing factors accumulate during seed germination in double CBC mutant in the presence of ABA

To reveal significant differences in transcript abundance between the double mutant and the single *hvcbp20.ab* and *hvcbp80.b* mutants, together with the WT in response to ABA, analyses were performed at the transcript level by examining the DET. The highest number of DETs (3404) was exclusively expressed in the double mutant. In the other genotypes, 1188 DETs were exclusive to *hvcbp20.ab*, 2282 to *hvcbp80.b* and 1192 to the WT (Fig. 3c). As the CBC complex is involved in RNA metabolism, including AS, the analysis of isoforms in *hvcbp20.ab/hvcbp80.b* will suggest the roles of the CBC in the response to ABA (Supplementary Fig. S3). The double mutant exhibited the highest number of events across most features of AS transcripts, particularly the premature termination codons (PTC) and non-sense mediated decay (NMD) categories, suggesting a possible interaction between the *CBP20* and *CBP80* genes in response to ABA treatment. GO enrichment analysis showed that genes encoding exclusively upregulated transcripts (DET) in double mutant are involved in GO 'biological process' (GO-BP) that involve metabolism and genes downregulated involve transcripts engaged in small molecule metabolic processes and anion and ion transport (Fig. 3d; Supplementary Data S11).

Furthermore, to quantify the modifications in the AS machinery attributable to the double mutation, transcripts linked to RNA splicing (GO:0008380) were catalogued among the uniquely expressed DETs in the double mutant and compared to the single mutants and WT. This led to the identification of 61 genes that accounted for 72 unique DETs in the double mutant, a notable enrichment that may signify substantial reprogramming of the splicing landscape, indicative of an adaptive response to ABA (Fig. 3e; Supplementary Data S12).

## Predicted interactions of CBC complex subunits with RNA metabolism and splicing factors

To test whether the proteins encoded by the DEGs and DAS genes, identified specifically for *hvcbp20.ab/hvcbp80.b* embryos in the presence of ABA, demonstrated direct physical interactions with the CBC subunits, a putative model of protein–protein interaction (PPi) was devised using the STRING database (Fig. 4, Supplementary Data S13). Utilizing CBP20 and CBP80 as seeds in this analysis, our search revealed 28 proteins that exhibited physical interactions with CBC subunits, as corroborated by the experimental data deposited in STRINGdb. A breakdown of these interactions revealed that 20 proteins interacted with both CBP20 and CBP80, while four interacted only with the CBP20 subunit, and another four with CBP80 (Supplementary Data S14). Intriguingly, within this interactome, seven genes were classified as DEGs and two as DAS, identified specifically in *hvcbp20.ab/hvcbp80.b*. These genes predominantly function in RNA processing and splicing, thereby highlighting the interconnectedness between these processes and the CBC complex. Notably, these DEGs are genes encoding SF, including arginine/serine-rich 4 (BaRT2v18chr1HG011980), which interacts with both CBC subunits. Moreover, two splicing factors that interact solely with CBP20 are noteworthy: the splicing factor U2af small subunit A (U2AF35A; BaRT2v18chr5HG254110) and the CELL DIVISION CYCLE 5-like protein (CDC5; BaRT2v18chr5HG272090) as detailed in Supplementary Data S14. These results showed an association between the CBC complex and other splicing and RNA processing factors, confirming its involvement in the regulation of seed germination via AS. The proteins encoded by these genes might act as CBC-dependent regulators of seed germination in the presence of ABA.

## Brassinosteroid biosynthesis may be increased in *hvcbp20.ab/hvcbp80.b* mutant in the presence of ABA

Alterations in BR signaling were observed in the double mutant through transcriptome profiling. Gene ontology enrichment analysis of genes specifically upregulated in double mutant demonstrated that the 'negative regulation of BR signaling' was overrepresented as the top Biological Process of GO categories. Additionally, genes encoding negative regulators of BR signaling were exclusively downregulated in the double mutant in response to ABA. To determine whether the ABA-insensitive phenotype also involves changes in BR biosynthesis, we analyzed distinctive the metabolomic profiles of germinating embryos from all genotypes under ABA treatment (Fig. 5a). The metabolomes were interrogated, and the sources variation were identified. Focusing on those sources of variation linked to hormones, this revealed significantly increased levels of 6-alpha-hydroxy-6-deoxycastasterone, a brassinolide biosynthesis intermediate, in the double mutant when compared to the WT and other genotypes. This upregulation aligns with the observed germination patterns, in which the double mutant demonstrated an enhanced germination rate, suggesting a growth-promoting effect and diminished ABA sensitivity (Fig. 5b).

## Towards Sebastian RTD: capturing genotype-specific transcriptome variations

To assess whether the BaRTv2.18 transcriptome[51], based on the Barke genotype, potentially overlooked transcriptomic data specific to the Sebastian genotype, adhered to the BaRTv2.18 assembly process the Reference Transcriptomic Dataset (RTD) named BarkeRTD was generated. This included assembling reference transcript datasets (RTDs) from Illumina RNA-seq short reads (RNAseq RTD) and PacBio Iso-seq long reads (Isoseq RTD comprised eight samples from this study representing each genotype under control and ABA), by using the same Barke reference genome as outlined by Ref.[51]. RNAseq RTD and Isoseq RTD were merged to produce a unified BarkeRTD. Comparative analysis indicated that BaRTv2.18 exhibits slightly higher genome coverage than BarkeRTD. This was due to the mapping of the Sebastian sequencing data to the Barke reference genome, potentially leading to the omission of Sebastian-specific genes[52]. However, BarkeRTD demonstrated notable augmentation, featuring approximately 5000 additional genes and 9000 more transcripts (Supplementary Table 2).


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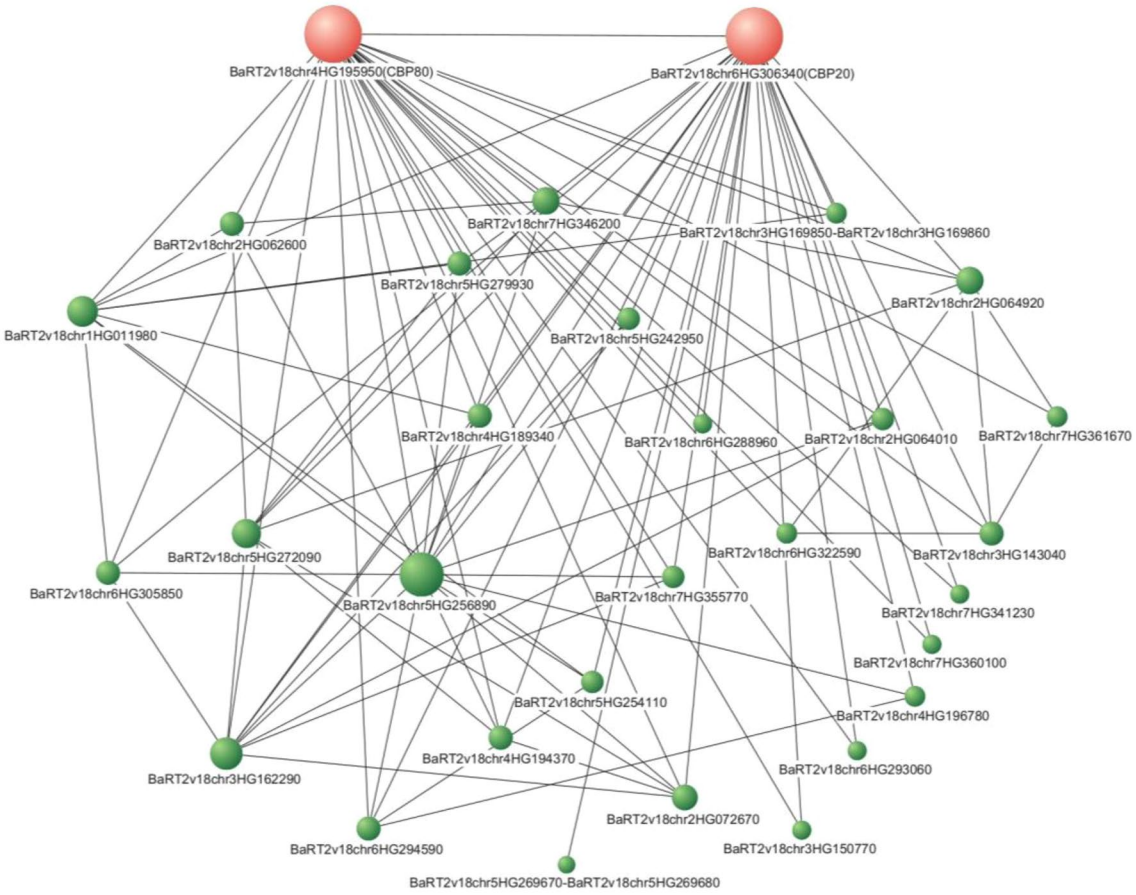


**Figure 4.** Network of protein–protein in silico physical interaction with CBP20 (red) and CBP80 (red) among differentially expressed genes (DEG) and genes with differential alternative splicing (DAS) in *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b* and WT after 75 µM ABA treatment compared to control conditions.

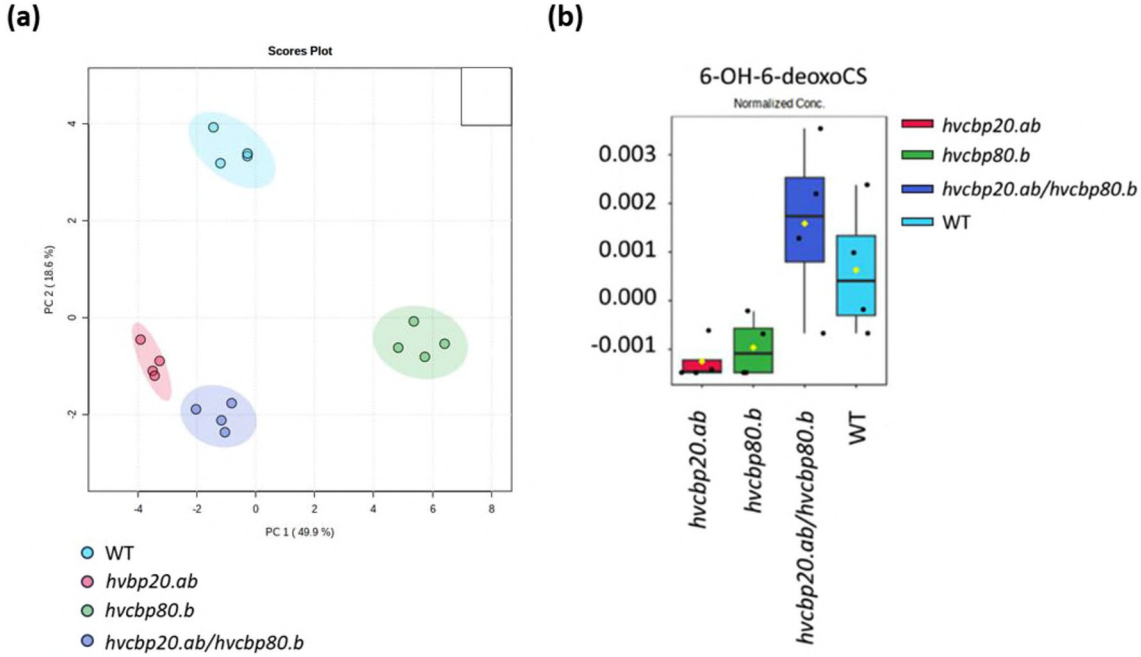


**Figure 5.** Metabolome profiling analysis of germinating embryos of *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b* and WT after 75 µM ABA treatment. (**a**) Principal component analysis (PCA) score plot of the metabolic profiles. (**b**) Endogenous 6-alpha-hydroxy-6-deoxycastasterone (6-OH-6-deoxoCS) content.


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The integration of RNAseq and Isoseq RTDs resulted in a substantial contribution, introducing over 12,000 novel splice junctions and 25,700 novel intron combinations to enrich the BarkeRTD, surpassing the content available in BaRTv2.18 (Fig. 6a,b). It is worth to note that none of the tissues used to produce BaRTv2.18 represent germinating barley embryos and we therefore prepared an RTD, also against Barke, that utilised our sequence data to provide sequence information that may be additional to BaRTv2.18. Our commitment to enhancing the dataset continues with the ongoing generation of new Iso-seq data. We are optimistic about achieving an even more comprehensive and refined Sebastian RTD using a Sebastian-based reference genome, coupled with additional sequencing data in the future.

## Discussion

Mutations in *CBP20* and *CBP80* confer hypersensitivity to ABA during seed germination in Arabidopsis and barley (*Hordeum vulgare*)[43–48]. Therefore, we expected the same ABA phenotype for the *hvcbp20.ab/hvcbp80.b* double mutant. However, our results demonstrated that the germination rate of the double mutant was comparable to that of the wild type (WT) in the presence of ABA, which is in striking contrast to the inhibition of germination in single mutants.

In Arabidopsis, the *cos1 coi1-2* double mutant exhibits resistance to pathogens and a deviation in senescence patterns[53], mirroring the phenomenon described in this study in the double mutant *hvcbp20.ab/ hvcbp80.b*. A reversal of the effect of the mutation in the *da3-1* single mutant, which leads to curly leaves and increased organ sizes, was observed in *sud1-1 da3-1* and *sud2-1 da3-1* double mutants[54]. Similarly, the growth of *bri1–5/ bri1–1D*, *bri1–5/brs1–1D* and *bri1–5/bak1–1D* double mutants is partially restored compared to the dwarf *bri1–5* single mutant with the mutation in the *BRASSINOSTEROID-INSENSITIVE 1* (*BRI1*) gene from the BR signal transduction pathway[55].

In all these examples, the double mutants complemented the genetic relationship, presenting a reversion to a WT-like phenotype that was not observed in the single mutants. This could imply a buffering effect or compensatory genetic interaction, where the confluence of two mutations mitigates the sensitivity observed in the single mutants. We cannot rule out a scenario in which the two subunits of the CBC can interact when both are mutated, while the interaction between mutated CBP20 and WT CBP80 or vice versa is not efficient. However, further experimental validation is required to confirm this. The double mutant insensitivity to ABA also suggests compensatory transcriptional reprogramming, possibly through altered expression or function of key genes within the ABA response network. This hypothesis was supported by the elevated transcriptional activity of the double mutant. Comparative transcriptome analysis revealed that the double mutant exhibited the most significant changes after ABA treatment, with a 30% increase in differentially expressed genes and transcripts relative to other genotypes. The prominence of differentially alternatively spliced genes in the double mutant suggests a reconfiguration of the splicing landscape, potentially contributing to the observed phenotype. The action of the CBC complex is notably more important in response to stress or ABA, evidenced by the minimal transcriptomic changes observed in the studied mutants compared to the WT under control conditions. These findings align with previous studies on the CBC complex in barley and Arabidopsis under stress conditions[43,56].

In plants, the involvement of the CBC complex in the constitutive and alternative splicing of pre-mRNA has been documented, where the CBC, together with the zinc finger protein SERRATE (SE), acts as a platform for interaction with other splicing factors[37,38]. Thus, we hypothesized that the role of CBC in seed germination may be splice-dependent. RNA-seq analysis of the embryo transcriptome in response to ABA revealed that genes and transcripts encoding splicing factors were specifically downregulated in the double mutant compared to *hvcbp20.ab, hvcbp80.b,* and WT. Among the DEG, we identified six genes encoding splicing factors that were upregulated only in the double mutants in the presence of ABA. One of them was *CELL DIVISION CYCLE 5* (*CDC5*) which encodes a Myb-related protein that is a subunit of the nuclear MOS4-ASSOCIATED COMPLEX (MAC) in Arabidopsis, important for the regulation of plant innate immunity[57]. Interestingly, CDC5 interacts with SE

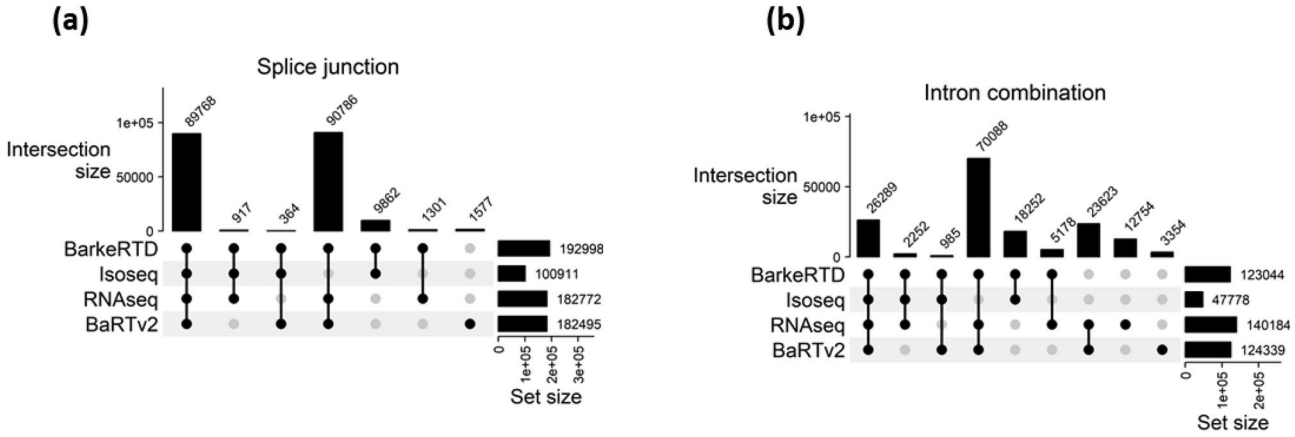


**Figure 6.** Comparisons of BarkeRTD, Isoseq RTD, RNAseq RTD and BaRTv2.18 on (**a**) splice junctions and (**b**) intron combinations of multi-exon transcripts. Splice junctions or Intron combinations shared by multiple transcripts were only counted once.


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and DICER-LIKE 1 (DLC1) during miRNA biogenesis and mediates alternative splicing of RESISTANCE TO RALSTONIA SOLANACEARUM 1 (RRS1) and RPS4[58–60]. The interactome analysis revealed a physical interaction between CBP20 and CDC5. The next step is to validate the relationship between the CBC complex, CDC5, and SE in AS regulation during seed germination. The other identified splicing factor is the U2 auxiliary factor (U2AF), which is a part of the core of the spliceosome[61]. U2AF is a heterodimer composed of the U2AF35 and U2AF65 subunits. U2AF35 binds to the 3′ splice site, whereas U2AF65 binds to the polypyrimidine tract (PPT) of nascent pre-mRNA[62]. Moreover, U2AF35 undergoes alternative splicing to form two isoforms, U2AF35a and U2AF35b, which interact with U2AF65[63]. Other studies in Arabidopsis have shown that U2AF65a and U2AF65b interact with SPLICING FACTOR 1 (SF1)[64,65]. SF1 acts as a splicing enhancer and is a member of the serine-arginine-rich (SR) protein family that facilitates spliceosome assembly[64,66–69]. Several studies have revealed that SR proteins regulate plant responses to environmental stress via AS[70–73].

Our study suggests a physical interaction between CBP20 and U2AF35a (BaRT2v18chr5HG254110) based on interactome analysis and identified increased expression of the putative branchpoint-bridging protein-like (SF1; BaRT2v18chr2HG081650), suggesting its involvement in the regulation of seed germination. Another factor from the SR family identified in our study that probably interacts with both CBC subunits is an arginine/serine-rich 4 (BaRT2v18chr1HG011980) a homolog of AtRS2Z33. In Arabidopsis, ectopic expression of *AtRS2Z33* affects the AS of other genes, as well as the AS of its pre-mRNAs, generating splice variants containing premature termination codons (PTC), which are then directed into the nonsense-mediated decay (NMD) pathway. It ensures an important post-transcriptional mechanism linking AS with NMD and regulates cellular protein levels in the cells[74,75]. Taken together, the altered expression of these splicing factors exclusively in *hvcbp20.ab/hvcbp80.b* suggests their potential dependency on the entire CBC complex. In addition, the potential interaction between the subunits of the CBC complex and the homologs of AtCDC5, AtU2AF35b/AtU2AF35a, and AtRS2Z33 suggests their common role during germination in the presence of ABA, which should be further investigated. We speculate that the 1.5-fold higher amount of DAS in *hvcbp20.ab/hvcbp80.b* compared to *hvcbp20.ab*, *hvcbp80.b,* and WT is related to the altered expression patterns of the identified SFs.

Raczynska et al.[38] investigated young Arabidopsis seedlings and suggested that the entire CBC complex is involved in alternative splicing, with an emphasis on the first intron of the transcript. The CBP80 subunit plays a substantial role in alternative splicing, as demonstrated by the fact that the *Atcbp80/abh1* mutant and the double mutant displayed significantly more common changes in alternative splicing levels than the *Atcbp20* mutant and the double mutant. In our study, we did not observe such a relationship because there were 17 common DAS genes for *hvcbp20.ab* and the double mutant and 10 for *hvcbp80.b* and the double mutant. Most DAS genes (94 DAS) were exclusive to *hvcbp20.ab/hvcbp80.b*, of which 76 DAS (81%) contained DTU transcripts. This indicated the importance of the entire barley CBC complex interaction in AS regulation during the seed germination stage.

Our approach, focusing on gene and transcript level expression changes, aimed to comprehensively investigate how the CBC influences alternative splicing dynamics, resulting in the generation of diverse transcript isoforms. This allowed us to observe the effect of CBC on AS not only at the gene level but also directly at the transcript level. Mutations in both CBC subunits led to a significantly increased accumulation of SF transcripts in the *hvcbp20.ab/hvcbp80.b* mutant. Among the 72 identified DET in SF, the presence of isoforms encoding homologs of AtSE, AtPRP4KA, AtPININ, and AtSR45 were particularly interesting. We observed the accumulation of two SF-coding transcripts (BaRT2v18chr7HG374470.5, BaRT2v18chr7HG374470.3), specifically in *hvcbp20.ab/hvcbp80.b*. Recent studies have shown that SE is also regulated by phosphorylation by the pre-mRNA PROCESSING FACTOR 4 KINASE A (PRP4KA), affecting its protein activity and proper accumulation in cells[76]. The *PRP4KA* transcript (BaRT2v18chr5HG220870.3) was also strongly upregulated in *hvcbp20.ab/hvcbp80.b* embryos in the presence of ABA. These results suggest a potential relationship among CBC, SE, and PRP4KA in fine-tuning germination in response to ABA, which requires further evaluation. We detected increased expression of two transcripts of the *AtSR45* homolog (BaRT2v18chr3HG170170.2 and BaRT2v18chr3HG170170.8), which is a highly conserved RNA-binding protein. SR45 interacts with the ABA-dependent splicing factors SNW/SKI-INTERACTING PROTEIN (SKIP) and SUA, and approximately 30% of the transcripts that bind to SR45 are involved in the ABA-signaling pathway[77,78]. Moreover, SR45 interacts with other SFs, including U2AF35b and its paralogs U2AF35, RSZ21, and SR34a, which were upregulated in our study (BaRT2v18chr3HG143420.7, BaRT2v18chr6HG321740.4, BaRT2v18chr6HG321740.5, and BaRT2v18chr2HG059400.6)[79–82]. This suggests the existence of an ABA-dependent interaction network that facilitates spliceosome assembly at the seed germination stage, in which the CBC complex is involved.

The upregulation of specific transcription factors in the double mutant further suggests a broad regulatory capacity, with the potential to reshape the ABA response during the germination stage. The highest number of TFs identified in *hvcbp20.ab/hvcbp80.b* corresponded to the largest changes in DEG and DET levels in this mutant after ABA treatment. Five of the exclusively expressed TFs were particularly interesting because they potentially bind to the promoters of the splicing factors *AtCDC5*, *AtEMB2016*, *AtSF1*, *AtRS2Z33*, *AtU2AF35b*/*AtU2AF35a* and *AtSUA*. These TFs are likely to also regulate *AtREM4.1* and the SAPK10 homolog of *AtSnRK2*s is important for ABA and BRs signaling.

ABA and BR antagonistically regulate various plant developmental processes, including seed germination[23,83]. A particularly interesting finding was that the 'negative regulation of brassinosteroids' was within the most significant biological process (BP) in the GO category in germinating embryos of double mutant in the presence of ABA. Interestingly, negative regulators of BR signaling were specifically downregulated in the double mutant (Supplementary Data S5). We detected reduced expression of *REMORIN* (REM4.1; BaRT2v18chr2HG069620) and *BRI1 KINASE INHIBITOR 1* (BKI1; BaRT2v18chr 5HG251390). In Arabidopsis, insertional mutants in *REM4.1* exhibit strong insensitivity to ABA during seed germination[84]. This mutant was even more insensitive than the *abi1-1 (abscisic acid-insensitive 1–1)* mutant[85]. When the level of ABA in the cell increases, *OsREM4.1* is repressed by OsbZIP23 (OsABF4). Subsequently, OsREM4.1 inhibits BR signaling by disrupting the complex


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formation of OsBRI1 and OsSERK1[86]. In our study, we did not identify a homolog of the bZIP23 transcription factor among exclusive DEGs in *hvcbp20.ab/hvcbp80.b*. However, we observed reduced expression of the transcription factor TGACG MOTIF-BINDING FACTOR 6 (TGA6; BaRT2v18chr1HG033440), which belongs to the basic leucine zipper (bZIP) gene family. TGA6 contains a TFBS in the promoter sequence of *REM4.1*, thus potentially regulates *REM4.1*.

BRI1 INHIBITING KINASE 1 (BKI1), such as REM4.1, interferes with the formation of the BRI1 complex with BRASSINOSTEROID RECEPTOR INSUSPENSIVE KINASE 1 (BAK1)[87–90]. Therefore, it is possible that REM4.1 and BKI1 act as independent negative regulators of BR signaling[86].

In addition, among the specifically downregulated genes in the presence of ABA in *hvcbp20.ab/hvcbp80.b*, we observed the gene encoding the serine/threonine-protein kinase SAPK10 (SAPK10; BaRT2v18chr7HG385230), which is highly homologous to SnRK2s in Arabidopsis. ABA induces *OsSAPK10* and its overexpression confers hypersensitivity to ABA[91]. In plants, SnRK2 kinases physically bind and phosphorylate ABF to transmit ABA signals[92]. Moreover, our interactome analyses showed that upregulated transcription of TF from the CYSTEINE-RICH POLYCOMB-LIKE PROTEIN (CPP) family (BaRT2v18chr1HG048310) that potentially binds to SAPK10. To understand the regulatory mechanism, we need to confirm whether the transcription factor CPP interacts with *SAPK10 in planta* and whether SAPK10 adds a phosphate group to TGA6, further influencing *REM4.1*. Additionally, the metabolome results showed increased concentrations of castasterone, which is a precursor to brassinolide (BL), the most active BR[93,94], in germinating embryos of *hvcbp20.ab/hvcbp80.b* and WT plants in the presence of ABA. Considering these results, we speculate that the downregulation of *REM4.1* and *BKI1* genes from the BR signaling pathway in the presence of ABA, together with potentially increased BR biosynthesis, resulted in the promotion of seed germination in the double *hvcbp20.ab/hvcbp80.b* mutant. Our findings demonstrate that the disruption of both CBC subunits potentially impairs the crosstalk between ABA and BR signaling and may induce BR biosynthesis. However, further research is required to understand the biological and genetic bases of these observations. We propose a hypothetical model of action for the identified factors in *hvcbp20.ab/hvcbp80.b* explaining its seed germination phenotype in the presence of ABA linked to signaling (Fig. 7).

In conclusion, our investigation demonstrated that the barley double mutant *hvcbp20.ab/hvcbp80.b* exhibits resistance to ABA during seed germination, suggesting that simultaneous mutations in the CBC subunits can mitigate ABA sensitivity through genetic interactions. Our detailed transcriptomic analysis of ABA responses highlights significant transcriptional reorganization, marked by shifts in gene expression, alternative splicing, and transcript isoform variation. The distinct phenotypes of the double mutant likely arise from the activation of specific gene and transcript sets depending on both CBC subunits. This study is the first to establish the crucial role of CBC in ABA-mediated seed germination in barley, particularly through its influence on alternative splicing and its interplay with the ABA and BR signaling pathways. Moreover, we have identified several key components of signaling pathways dependent on CBC, and thus warrant further investigation, which paves the way for future studies to comprehensively decode CBC function in ABA signaling during germination.

## Materials and methods

### Plant material

We used grains and embryos of barley TILLING mutants: the *hvcbp20.ab,* in the *CBP20* gene (*CAP-BINDING PROTEIN 20*; BaRT2v18chr6HG306340; Refs.[43,95]), and *hvcbp80.b*, in the *CBP80* gene (*CAP-BINDING PROTEIN 80*; BaRT2v18chr4HG195950), single mutants, the *hvcbp20.ab*/*hvcbp80.b* double mutant, and the 'Sebastian' wild-type (WT), all sourced from the *Hor*TILLUS population[96]. Single mutants were identified through TILLING in the $M_2$ generation (then backcrossed with WT to clean the genetic background), and the double mutant was identified via genotyping the $F_2$ progeny following crossbreeding of the single mutants (Supplementary Fig. S1). Mutations in the *CBP20* and *CBP80* genes in *hvcbp20.ab/hvcbp80.b* double mutant were confirmed using Sanger sequencing in three biological replicates in two consecutive generations of double mutant after self-pollination series.

### Seed germination assay in the presence of 75 µM ABA, embryos isolation and RNA extraction

Seeds were sterilized in 20% sodium hypochlorite solution for 20 min and washed with sterilized water for 5 min. They were then placed in Petri dishes with three layers of filters and treated with either sterilized water or sterilized water containing 75 µM ABA (cis–trans-abscisic acid; catalog no. 862169; Sigma-Aldrich). The seeds were stratified for 4 d at 4 °C and then transferred to a growth chamber. Germination was defined as the visible emergence of the radicle through the seed coat and was assessed on 1 and 7 DAI (days after imbibition). The assay was performed in three biological replicates, each comprising 30 seeds of each genotype per petri dish. At 1 DAI, the embryos were isolated from the endosperm using a sharp scalpel blade, placed in microcentrifuge tubes (Eppendorf) containing RNAlater reagent, and stored at 4 °C until RNA isolation. RNA from isolated germinating embryos at 1 DAI was extracted using the mirVana™ Isolation Kit (Ambion, USA) following the manufacturer's instructions. RNA was isolated in four biological replicates, each consisting of 20 embryos. In total, 32 RNA samples were extracted. RNA concentration and quality were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA) and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA).

### Library construction and Illumina sequencing

In total, 32 RNA samples (30 µL each) obtained from the germinating embryos at 1 DAI of four tested barley genotypes ('Sebastian' (wild-type), *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b*), each in four biological replicates, subjected to two treatment conditions (control and 75 µM ABA) at 1DAI, were used for TruSeq stranded mRNA cDNA library construction and next-generation sequencing (NGS) (Macrogen Inc., South Korea). Illumina RNA-seq was performed using a NovaSeq6000 sequencer (40 M, 2xPE 150 bp). Preprocessing of the raw


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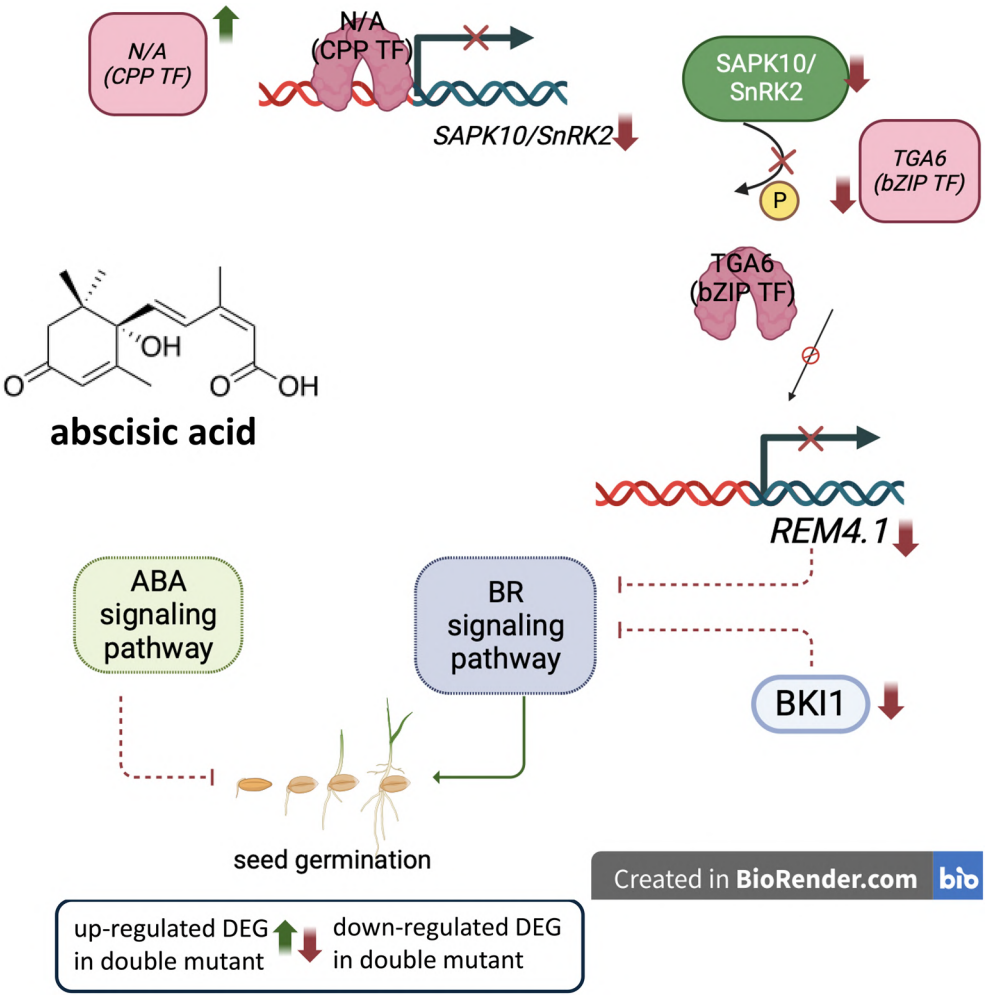


**Figure 7.** Hypothetical model explaining the seed germination phenotype of the *hvcbp20.ab/hvcbp80.b* in the presence of ABA linked with BR signaling. In the presence of ABA in the *hvcbp20.ab/hvcbp80.b* double mutant, the unknown transcription factor from the CPP-family is exclusively upregulated. This transcription factor potentially binds *SAPK10* and inhibits its expression. It leads to reduced phosphorylation of the TGA6 transcription factor by SAPK10 and reduced expression of the *REM4.1* gene encoding a negative regulator of BR signaling. Additionally, in *hvcbp20.ab/hvcbp80.b,* in response to ABA, the *BKI1* gene encoding the negative regulator of the BR signaling pathway is exclusively downregulated. All this together causes the seed germination process in the *hvcbp20.ab/hvcbp80.b* double mutant to be inhibited in the presence of ABA. The dashed lines represent possible interactions. The solid lines represent known interactions. The green color indicates exclusively differentially upregulated genes in *hvcbp20.ab/hvcbp80.b* in response to ABA. The red indicates exclusively differentially downregulated genes in *hvcbp20.ab/hvcbp80.b* in response to ABA. P-phosphorylation. Illustration created with BioRender.

reads was performed using FastQC, adapters were removed with Cutadapt, and quality control of the trimmed reads was checked again with FastQC[97]. Low-quality reads were filtered using Cutadapt software. Paired-end reads were mapped to the BaRTv2.18[51] barley reference transcriptome using the Kallisto tool[98]. The mapped reads were quantified and normalized to transcripts per million (TPM) using Kallisto.

## Differential expression analysis

Differential gene and transcript expression analyses were performed using the limma-voom package in a 3D RNA-seq application[99]. Analysis was performed for two treatment groups (ABA and control) and seven comparisons (contrast groups): under control conditions between the *hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b* mutants against the 'Sebastian' parental variety (control.cbp20-control.WT, control.cbp80-control.WT, control.double-control.WT) and after ABA treatment of each genotype (*hvcbp20.ab*, *hvcbp80.b*, *hvcbp20.ab/hvcbp80.b* and wild type 'Sebastian') against control conditions (ABA.WT-control.WT, ABA.cbp20-control.cbp20, ABA.cbp80-control.cbp80, ABA.double-control.double). In all contrast groups, differentially expressed genes (DEG), which show significant changes in expression levels between tested conditions; differentially expressed transcripts (DET), specific transcripts of genes exhibiting significant expression changes compared to other transcripts


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within the same gene; differential alternative splicing (DAS), where genes with multiple transcript isoforms show varying abundance patterns between conditions; and differential transcript usage (DTU), highlighting specific transcript variants preferentially expressed between tested conditions, were analyzed. The threshold for significant differential gene and transcript expression was $\log_2FC \geq 1.5$ or $\leq -1.5$, with a Benjamini-Hochberg (BH) adjusted p-value < 0.01. To identify significant DAS, the percentage spliced (ΔPS) threshold was set at 0.5. The DAS genes within each contrast group were identified based on an adjusted p-value below the predefined threshold (< 0.01) and the presence of at least one transcript exhibiting a ΔPS greater than 0.5. For DTU identification, each transcript's expression was compared to the weighted average expression of all other transcripts within the same gene. A transcript was classified as DTU if its adjusted p-value was below the predefined threshold (< 0.01) and the absolute value of ΔPS exceeded the defined cut-off value of 0.5. To identify common DEGs and DETs between all tested genotypes, and exclusive for each genotype in the treatment groups, Venn diagrams were constructed using the Venny 2.1 tool (https://bioinfogp.cnb.csic.es/tools/venny/). In this study, we focused on the genes and transcripts specific for *hvcbp20.ab/hvcbp80.b*. Gene Ontology enrichment analysis was performed using TopGO (version 2.50.0) implemented in R (version 4.2.1) with an adjusted p-value < 0.01. The BH method was used to correct the False Discovery Rate (FDR).

## Transcription factors (TFs) identification and binding site prediction

The PlantRegMap tool was used to predict transcription factors (TFs)[100]. Promoter sequences, defined as the 1500 bp upstream flanking regions, were retrieved through EnsemblPlants v. 45 using the BioMart tool, based on the MorexV3 barley genome version integrated into the EnsemblPlants database[101]. These promoter sequences were analyzed for transcription factor-binding sites (TFBS) using the PlantRegMap Binding Site Prediction feature. Subsequently, datasets detailing TFs and their potential target genes were merged to explore the potential regulatory interactions.

## Identification of Arabidopsis homologs

The BLAST tool within the Ensembl Plants database was used to identify Arabidopsis homologs (version of the genome TAIR10). A threshold of at least 40% similarity between sequences was set for homology[102].

## Analysis of interactome

Using the STRING database (version 11.5), a predicted network of physical protein–protein interactions (PPIs) was constructed for the DEGs and DAS genes in the *hvcbp20.ab/hvcbp80.b* mutant in ABA presence. For network analysis, the physical subnetwork was selected, and the default parameters were used with a medium confidence interaction score threshold of 0.4 and no additional interactors. The PPi network was visualized using Cytoscape software[103].

## Co-expressed genes analysis

All significantly differentially expressed genes (DEGs) identified within the RNA-seq experiment of germinating embryos at 1 DAI from tested mutants and WT were used to identify gene co-expression clusters. The average transcript per million (TPM) gene values of four biological replicates of the ABA-treated samples were used in the 'clust 1.10.8' tool in Linux with default settings was employed for this purpose. Heatmaps were generated using the R package 'Pheatmap' v.1.0.12, based on TPM values.

## Prediction of splicing factors

Splicing factors (SF) were searched by filtering the gene ontology (GO) term 'RNA splicing' (GO:0008380) from a list of exclusively differentially expressed genes (DEG) and a list of exclusively differentially expressed transcripts (DET) in each genotype studied under ABA vs. control conditions.

## Metabolome analysis

Embryos extracted from germinating seeds were homogenized in liquid nitrogen and subsequently chilled on ice. Precise 40 ± 1 mg of this tissue was then transferred into microcentrifuge tubes, followed by the addition of 1 mL of chloroform: methanol: $dH_2O$ mixture (1:2.5:1). The samples were vigorously mixed using by vortexing at 4 °C for 15 min before being placed back on ice. Afterward, they were centrifuged at 4 °C at 5000×*g* for 3 min. The supernatants, which contained both polar and nonpolar metabolites, were carefully transferred to new tubes and dried using a Buchi Rotavapor system at 25 °C, to avoid complete evaporation. Finally, 100 µL of the supernatant was reserved for metabolomic profiling using liquid chromatography-tandem mass spectrometry (LC–MS/MS) as described by Baptista et al.[104].

## PacBio sequencing and reference transcriptome dataset (RTD)

Eight RNA SMRTbell Iso-seq libraries were prepared using RNA extracted from the same samples as those used for Illumina sequencing but pooled for each genotype. Further libraries were sequenced using the PacBio sequel II platform according to the manufacturer's protocol (Macrogen Inc., Seoul, Republic of Korea). To investigate whether transcriptomic analysis specific to the Sebastian genotype was overlooked using BaRTv2.18, a Barke-based transcriptome, and assess the potential necessity of assembling a Sebastian-specific reference transcript dataset (RTD) in future endeavors, we adhered to the BaRTv2.18 assembly pipelines, assembling RTDs from both Illumina RNA-seq short reads and PacBio Iso-seq long reads[51]. Specifically, the STAR mapping tool[105] was used to align the trimmed RNA-seq reads of the 32 samples to the Barke reference genome[106]. Transcript models were assembled from the read alignment using two assemblers: Stringtie[107] and Scallop[108]. The resultant transcripts in


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different samples, along with those from BaRTv2.18, were merged to generate an RNAseq RTD using RTDmaker (https://github.com/anonconda/RTDmaker). In addition, PacBio Iso-seq data were generated from four samples (a pool of RNA was used for this purpose per genotype analyzed). After pre-processing the Iso-seq data with Isoseq3 pipelines (https://github.com/PacificBiosciences/IsoSeq), full-length non-concatemer (FLNC) reads were mapped to the Barke reference genome using Minimap2[109]. Transcript models were assembled through TAMA, and transcript start and end sites, as well as splice junctions, were refined using the BaRTv2.18 approach[51], yielding Isoseq RTD. Leveraging the full-length transcript capability of PacBio, we integrated transcripts from the RNA-seq RTD that contributed to novel splice junctions or gene loci into the Isoseq RTD, resulting in a more comprehensive BarkeRTD. Comparative analyses of splice junctions and transcript-level intron combinations across BarkeRTD, Isoseq RTD, RNA-seq RTD, and BaRTv2.18, were conducted to ascertain whether BaRTv2.18 may have overlooked critical information about spliced transcripts.

## Statement regarding experimental research on plants

Experimental research on plants complied relevant institutional, national, and international guidelines and legislation.

## Data availability

The data generated and analyzed in this research article are included in all figures, tables, and supplemental data. The RNA-seq data used in the present study have been deposited into EMBL-EBI (EMBL's European Bioinformatics Institute) in the Array Express repository (https://www.ebi.ac.uk/) under accession number E-MTAB-13989.

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## Author contributions

ADG conceptualization; ADG, ES experimental design; ADG, ES, research analysis; AC, WG, CGS PacBio sequencing analysis, BH, LM, MB metabolome analysis; ES investigation and data interpretation, ADG data curation; ADG, ES writing–original draft; ADG, ES writing–review and editing; ES visualization; ADG supervision; ADG project administration; ADG funding acquisition. All authors reviewed the manuscript.

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## Competing interests

The authors declare no competing interests.

## Additional information

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**Correspondence** and requests for materials should be addressed to A.D.-G.

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## Publikacja 3

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# Mapping the molecular signature of ABA-regulated gene expression in germinating barley embryos

Ewa Sybilska[1], Bahareh Sadat Haddadi[2], Luis A. J. Mur[2], Manfred Beckmann[2], Szymon Hryhorowicz[3], Joanna Suszynska-Zajczyk[3,4], Monika Knaur[3], Andrzej Pławski[3,5] and Agata Daszkowska-Golec[1*]

## Abstract

**Background** Abscisic acid (ABA) regulates key plant processes, including seed germination, dormancy, and abiotic stress responses. While its physiological role in germination is well-documented, the molecular mechanisms are still poorly understood. To address this, we analyzed transcriptomic and metabolomic changes in ABA-treated germinating barley (*Hordeum vulgare*) embryos. To map ABA-responsive gene expression across embryonic tissues, we employed the Visium Spatial Transcriptomics (10× Genomics). This approach, which remains technically challenging to be applied in plant tissues, enabled the precise localization of gene expression across six embryo regions, offering insights into tissue-specific expression patterns that cannot be resolved by traditional RNA-seq.

**Results** Transcriptomic analysis indicated that ABA acts primarily as a germination repressor. Gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses linked ABA-inhibited genes to energy metabolism, lignin biosynthesis, cell wall organization, and photosynthesis, while induced genes were associated with environmental adaptation and phytohormone signaling. Differentially expressed genes (DEGs) correlated with metabolites involved in phytohormone pathways, including gibberellins, jasmonates, brassinosteroids, salicylic acid, auxins, and ABA metabolism. Comparisons with developing seed transcriptomes suggested an ABA-associated gene expression signature in embryos. Spatial transcriptomics technique made possible the precise identification of ABA-induced transcriptional changes within distinct embryonic tissues.

**Conclusions** Integrating transcriptomics, metabolomics and spatial transcriptomics defined the molecular signature of ABA-induced modulation of phytohormonal crosstalk, energy metabolism, and tissue-specific gene activity in germinating seeds. The successful use of spatial transcriptomics adds a novel layer of resolution for understanding tissue-specific ABA responses during barley seed germination. These findings offer new insights into the ABA role in seed germination and potential strategies for enhancing crop resilience.

**Keywords** ABA, Barley, Embryo, Metabolomics, Seed germination, Spatial transcriptomics, Transcriptomics

*Correspondence:
Agata Daszkowska-Golec
agata.daszkowska@us.edu.pl
[1]Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Katowice, Poland
[2]Department of Life Science, Aberystwyth University, Aberystwyth, UK
[3]Institute of Human Genetics, Polish Academy of Sciences, Poznan, Poland
[4]Department of Biochemistry and Biotechnology, Poznan University of Life Sciences, Poznan, Poland
[5]Department of General, Endocrinological Surgery and Gastroenterological Oncology, Poznan University of Medical Sciences, Poznan, Poland

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## Background

Abscisic acid (ABA) is a key regulator of seed dormancy, preventing premature germination under unfavorable conditions [1, 2]. In order to maintain dormancy, ABA synthesized *de novo* in the embryo plays a crucial role, but ABA produced in maternal tissues only plays a contributory role [3, 4]. The suppressive action of ABA is closely linked to its agonistic role with gibberellins (GA). ABA inhibits the expression of GA biosynthetic genes whilst strengthening the endosperm cell wall to delay germination [5]. In Arabidopsis, mutants defective in ABA biosynthesis or signaling exhibit germination rate, whereas GA biosynthesis mutants do not germinate in the absence of exogenous GA [6–10]. The balance between ABA and GA is controlled by a complex transcriptional network. ABSCISIC ACID INSENSITIVE 3 (ABI3) and ABI5 transcription factors regulate the expression of the *MOTHER OF FT AND TFL1* (*MFT*) genes through a negative feedback loop mechanism in the ABA signaling pathway [11]. Importantly, crosstalk between ABA and other phytohormones is also crucial for controlling seed germination and dormancy. Auxins increase ABA levels and inhibit GA synthesis, which delays germination [12, 13]. However, the effect of auxins on seed germination appears to be dose-dependent. High auxin concentrations promote dormancy, whereas low auxin concentrations promote germination [14]. Ethylene (ET) reduces ABA accumulation by both inhibiting its synthesis and promoting its inactivation, and by negatively regulating ABA signaling [15]. Brassinosteroids (BR) promote seed germination via an *MFT*-dependent pathway and regulate starch degradation in the endosperm by modulating α-amylase expression [16, 17]. BRASSINOSTEROID INSENSITIVE 2 (BIN2) kinase binds to the ABI5 protein and phosphorylates it to influence ABA signaling [18]. Salicylic acid (SA) has been shown to inhibit germination under normal conditions, whereas it supports germination under salt stress by reducing oxidative damage [19]. SA also inhibits germination in a dose-dependent manner [20]. Cytokinins (CTK) act antagonistically to ABA, reducing *ABI5* expression and promoting germination [21]. The role of jasmonate (JA) is complex. The JA precursor, oxylipin 12-oxo-phytodienoic acid (OPDA), enhances ABA signaling, while jasmonoyl-l-isoleucine (JA-Ile) reduces dormancy [22, 23]. However, the effects of JA may be species-dependent. In wheat, JA stimulates seed germination; however, in Arabidopsis, jasmonate ZIM-domain (JAZ) proteins inhibit the activity of ABI3 and ABI5 to reduce the ABA signal [24, 25]. Other reports have suggested that JA in combination with auxins supports ABA function, leading to the inhibition of seed germination [26, 27].

Despite the increasing use of technologies integrating transcriptome and metabolome data in plant research, detailed analyses of the regulatory mechanisms underlying ABA responses during seed germination remain limited [28]. These types of studies have shown that ABA affects germination by regulating sugar metabolism and the cell wall in rapeseed, inhibiting photosynthesis and secondary metabolism in pear, and inducing seed dormancy via the *NCED6* gene in Arabidopsis [29–31]. Additionally, new information can be obtained through such technologies as Visium Spatial Transcriptomics (10× Genomics), which allows for high-resolution mapping of gene expression in specific plant tissues. A recent study by Peirats-Llobet et al. (2023) demonstrated the potential of spatial transcriptomics in plant research, focusing on germinating seeds [32]. This study provides a detailed spatial map of gene expression during seed germination, uncovering key regulatory networks and tissue-specific transcriptional activities that govern this critical developmental process.

In this study, we integrated transcriptomic and metabolomic analyses to elucidate the effects of ABA on barley embryo germination. This multi-omics strategy revealed coordinated interactions between ABA and other phytohormones, pinpointing the key genes and metabolites involved in this crosstalk. By comparing the transcriptomes of ABA-treated embryos and developing seeds, we delineated a common ABA-responsive gene set and identified genes uniquely regulated during germination. Furthermore, spatial transcriptomics enabled us to surpass the limitations of bulk RNA-seq by precisely localizing ABA-influenced gene expression across distinct embryo tissues. Together, these provided the molecular signature of ABA effects during seed germination.

## Methods

### Plant material and ABA treatment conditions

In our study, we used the spring barley cultivar 'Sebastian' that was selected due to its high yield potential, good malting quality, resistance to lodging, and strong resistance to stem rust (*Puccinia graminis*) and leaf rust (*Puccinia hordei*). Previously, we used 'Sebastian' as a parent variety to create the *Hor*TILLUS population [33]. The initial seeds were obtained from HODOWLA ROŚLIN STRZELCE Sp. z o.o IHAR Group, Poland. Subsequently, seeds were multiplied in our laboratory and collected from plants grown in the greenhouse of the Institute of Biology and Biotechnology in Katowice, Poland.

Barley (*Hordeum vulgare*) embryos of the 'Sebastian' cultivar were isolated from germinating seeds in the presence of 75 µM abscisic acid (ABA) and under control conditions at 1 day after imbibition (DAI). This concentration of ABA had been previously optimized based on dose–response experiments, as it enables differentiation between ABA-sensitive and ABA-insensitive genotypes, as shown in our previous study [34]. The ABA treatment

started from sterilized seeds and continued until one day after imbibition (1 DAI) up to the moment of embryo isolation. Firstly, the barley seeds were surface-sterilized in a 20% sodium hypochlorite solution for 20 min, and rinsed thoroughly three times in sterile distilled water for 5 min per wash. Subsequently, the seeds were placed in 90 mm Petri dishes lined with three layers of Whatman filter paper and moistened with 5 ml of either sterile distilled water (control) or distilled water supplemented with 75 µM ABA (cis–trans-abscisic acid; Sigma-Aldrich, cat. 862169; Sigma-Aldrich). The seeds were stratified at 4 °C in the dark for four days to synchronize germination. After stratification, the Petri dishes were transferred to a growth chamber set to 22 °C, with a photoperiod of 16 h light / 8 h dark and a light intensity of 200 µmol $m^{-2}$ $s^{-1}$. Embryos were collected at 1 DAI and preserved in RNAlater™ Stabilization Solution (Thermo Fisher Scientific, cat. AM7020) until RNA isolation.

#### RNA extraction, cDNA library construction and sequencing

RNA was extracted from four biological replicates, each consisting of 20 'Sebastian' embryos isolated at 1 DAI under control conditions or in the presence of 75 µM ABA. Total RNA from each sample was isolated according to the manufacturer's instructions using the mirVana™ Isolation Kit (Ambion, USA). RNA concentration and quality were assessed using a NanoDrop spectrophotometer and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA library was constructed using the TruSeq stranded mRNA cDNA library preparation technique, followed by next-generation sequencing (NGS) at Macrogen Inc., South Korea. Sequencing was performed using an Illumina NovaSeq6000 system (40 million paired-end reads with a length of 150 bp). The initial quality assessment of the raw reads was performed using FastQC, and adapters were trimmed using the Cutadapt tool [35]. Quality control was re-evaluated after trimming. Poor-quality reads were removed using the Cutadapt software [35]. The cleaned paired-end reads were then aligned to the barley reference transcriptome BaRTv2.18 using Kallisto software [36, 37]. The mapped reads were quantified and normalized to transcripts per million (TPM) with Kallisto [36].

#### Identification of differentially expressed genes

Differential expression analysis was performed using the limma-voom pipeline in the 3D-RNA-seq platform [38]. The comparison was made between 'Sebastian' embryos under control conditions and those treated with ABA (ABA.WT vs. control.WT). Differentially expressed genes (DEGs) were identified based on a significance threshold of log2FC ≥ 1.5 or ≤ -1.5, with a p-value < 0.01, adjusted using the Benjamini-Hochberg method.

#### Gene function annotation

Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was performed using the TopGO package (version 2.50.0) in R (version 4.2.1) with an adjusted p-value threshold of < 0.01 [39]. The results were visualized in RStudio using the ggplot2 package (version 3.5.1) [40] (https://rstudio.com/). For the Kyoto En cyclopedia of Genes and Genomes (KEGG) enrichment analysis, enriched pathways were identified using the clusterProfiler package (version 4.10.1) in RStudio, with a corrected p-value cutoff criterion of < 0.01 [41]. The KO identifiers of the DEGs used as input were obtained using the BlastKOALA tool (https://www.kegg.jp/blastkoala/) by querying the KEGG ORTHOLOGY (KO) database. Plots were generated in RStudio using the ggplot2 package (version 3.5.1) [40] (https://rstudio.com/).

#### Prediction of transcription factors (TFs) and their binding sites

Transcription factors (TFs) were predicted using the PlantRegMap tool (https://planttfdb.gao-lab.org/prediction.php). Promoter sequences, corresponding to the 1500 bp regions upstream of the target genes, were extracted using the BioMart tool in EnsemblPlants v. 45, utilizing the MorexV3 barley genome version available in the EnsemblPlants database (http://plants.ensembl.org/biomart/martview/). Potential transcription factor-binding sites (TFBS) were targeted using the PlantRegMap Binding Site Prediction feature (https://plantregmap.gao-lab.org/binding_site_prediction.php). The resulting datasets of TFs and their associated target genes were integrated to assess the possible regulatory pairs.

#### Metabolome analysis

The metabolomic has been previously described by Sybilska et al. (2024) [34]. Embryos from germinating seeds were ground into fine powder using liquid nitrogen and then chilled on ice. Then, 40 ± 1 mg of tissue was transferred into microcentrifuge tubes, followed by the addition of 1 mL of chloroform: methanol: $ddH_2O$ mixture (1:2.5:1 v/v). The samples were thoroughly mixed by vortexing at 4 °C for 15 min, and then returned to ice. Subsequently, they were centrifuged at 5000×g for 3 min at 4 °C. The resulting supernatants containing polar and nonpolar metabolites were carefully collected in fresh tubes and dried at 25 °C using a Buchi Rotavapor system to prevent complete evaporation. A final volume of 100 µL was retained for metabolomic analysis via liquid chromatography-tandem mass spectrometry (LC-MS/MS), as previously described by Baptista et al. (2018) [42]. Partial Least Squares Discriminant Analysis (PLS-DA) was used with the holomics R package [43].

### Integrative transcriptomic and metabolomic analysis

Integrative omic assessments of transcriptomic data were undertaken using the holomics R package in RStudio (version 2023.12.0) [43] (https://rstudio.com/).

### Comparative transcriptome data assessments

Transcriptomic profiles generated in this study for germinating 'Sebastian' embryos were compared with the developmental expression in barley seeds described by Kovacik et al. (2024) [44]. Differentially expressed genes (DEGs) from our study and their BaRTv2.18 gene IDs were translated into their corresponding HORVU.MOREX identifiers. The BaRTv2.18 dataset is the most recent barley reference transcriptome, based on the Barke cultivar, whereas the HORVU.MOREX identifiers correspond to the older Morex reference genome [37]. Due to differences in reference datasets, in a small number of cases, BaRTv2.18 identifiers were mapped to the same HORVU.MOREX identifier. Thus, 3,621 DEGs (65%) were mapped to the corresponding HORVU.MOREX identifiers and used for cross-study analysis. Splice variants of the same gene were counted as a single DEG.

### Spatial gene expression analysis in germinating barley embryos

#### *Preparation of barley embryo sections*

From isolated (cv. Sebastian) embryos germinated under control conditions or with 75 µM ABA at 1 day after imbibition (DAI), the embryonic root was removed. Next, the embryos were placed in an optimal cutting temperature (OCT) medium and then frozen in an isopentane bath on dry ice. Frozen embryos were stored at − 80 °C, and then cut into 10 µm thick sections in a cryostat (Leica CM3050 S) at − 18 °C. The embryo sections were placed on the Visium Spatial Gene Expression Slide. Embryo RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA Integrity Number (RIN) was evaluated to determine the degree of RNA degradation using the Agilent 2100 Bioanalyzer. Slides were fixed in chilled methanol for 30 min at − 20 °C. After fixation, sections were stained for 5 min with 0.1% Safranin O (Sigma-Aldrich, cat. S8884-25G) in 50% ethanol. The sections were then washed in an alcohol series (50%, 70%, 100%) for 1 min. Slides were imaged in the bright field using a light microscope (Leica DS5500).

#### *Tissue optimization (TO) procedure*

To pre-permeabilize the tissue, the slides were assembled in a Visium slide cassette and incubated in pre-permeabilization solution (48 µl 10x Exonuclease I buffer (ThermoScientific, #EN0581); 4.5 µl of Bovine Serum Albumin (BSA), 10% Aqueous Solution, nuclease-free, Sigma-Aldrich, cat. 126615-25 ml,; and 2% (w/v) polyvinylpyrrolidone PVP40, Sigma-Aldrich, cat no. PVP40-500 g) at 37 °C for 30 min. This was followed by washing in 100 µl 0.1 × saline-sodium citrate (SSC) buffer (Sigma-Aldrich, cat. S6639L). The sections were permeabilized with Permeabilization mix™ (10x Genomics) at 37 °C for different times (2, 4, 6, 12, 18, and 24 min Tissue Optimization (TO) slides, including positive and negative control) or 6 min (Gene Expression (GE) slides). The wells were washed with 100 µl of 0.1× SSC buffer. After permeabilization, reverse transcription mixture™ (10x Genomics) was added to each section and incubated at 53 °C for 45 min, as described in the 10x Genomics User Guide (PN-1000186, CG000239_VisiumSpatialGeneExpression_UserGuide_RevD).

#### *Tissue removal and washes (TO slide only)*

To remove the tissue, a hydrolytic enzyme mixture was prepared by adding 70 µl of cellulase (Yakult -'ONOZUKA' R-10, cat. YAKL0012), pectate lyase (cat. E-PCLYAN2), and xyloglucanase (Megazyme, cat. E-XEGP), endo 1,4 β-xylanase (Megazyme, cat. E-XYNACJ), endo 1,4 β-mannanase (Megazyme, cat. E-BMACJ), and lichenase (Megazyme, cat. E-LICHN) to 140 µl of 250 mM sodium citrate (Sigma-Aldrich, cat. S-4641–1 kg). The enzymatic mixture was added to the wells, individual reaction chambers within the Visium Slide Cassette, and incubated in an IKA Mixer at 37 °C for 90 min with shaking (300 rpm). The wells were washed with 100 µl 0.1× SSC buffer. Samples were incubated with 10% $H_2O$ Triton X-100 solution (Sigma-Aldrich, cat. 93443-500 ml) in an IKA Mixer at 56 °C for 1 h with shaking (300 rpm), followed by a wash with 0.1× SSC buffer. Next wash consisted of a mixture of RLT buffer (Qiagen ref.79216) with 1% (v/v) β-mercaptoethanol, which was incubated in a Thermo Mixer at 56 °C for 1 h with shaking (300 rpm), followed by a wash with 0.1× SSC buffer. A final incubation with 70 µl proteinase K mixture (60 µl of proteinase K (Qiagen, cat. 19131), and 420 µl of PKD buffer (Qiagen, cat no. 1034963) was performed in a Thermo Mixer at 56 °C for 1 h with shaking (300 rpm). Hybridization chamber was detached, and the slide was washed in a Petri dish with 50 °C pre-warmed wash buffer 1 (2× SSC/0.1% sodium dodecyl sulfate (SDS) at 50 °C for 10 min with shaking (300 rpm). The slides were further washed with wash buffer 2 (0.2× SSC) and wash buffer 3 (0.1× SSC) at RT for 1 min with shaking (300 rpm). The slide was spin-dried in a swing-bucket centrifuge.

The tissue GE slide was then processed according to the Visium Spatial Gene Expression User Guide protocol.

### cDNA sequencing and differential gene expression analysis

cDNA sequencing was performed on a NovaSeq 6000 platform (Illumina) in paired-end mode with a read

length of 151 bp (Macrogen, The Netherlands). Read sequence analysis was performed with Space Ranger v3.1.0 using the barley reference genome, cv. MorexV3 [45] (https://www.10xgenomics.com/support/software/space-ranger/latest). Data visualization results were proc essed using Loupe Browser 8 (https://www.10xgenomics.com/support/software/loupe-browser/). Differential gene expression (DEG) analysis between the ABA-treated and control samples was performed in six clusters within the germinating embryo: coleoptile, cotyledon, mesocotyl, plumule, scutellum, and radicle. Genes with low average abundance were excluded, and only DEGs with p-value ≤ 0.05 and log2FC ≥ 0.25 were included in further analysis.

### Statistical analyses

Statistical analyses, including calculation of correlation coefficients and generation of plots, were performed using RStudio (version 2023.12.0) (https://rstudio.com/).

## Results

### ABA suppresses the expression of numerous genes in germinating embryos

To identify genes regulated by ABA during early seed germination, we analyzed transcriptomic changes in germinating embryos of the barley variety 'Sebastian' in the presence of 75 µM ABA versus control conditions at 1 DAI (Additional file 1: Data S1). A total of 5,533 differentially expressed genes (DEGs) were detected of which 3,533 (64%) were downregulated, while 2,000 (36%) were upregulated (Fig. 1A). Of the total, 2,715 DEGs exhibited low expression levels (TPM ≤ 1) under both control and ABA treatments but 1,595 genes (59%) showed reduced levels with ABA (Fig. 1B). Only 49 DEGs displayed high expression (TPM > 1000) in either treatment (Fig. 1C). Hence, ABA could be acting as repressor of gene expression during seed germination.

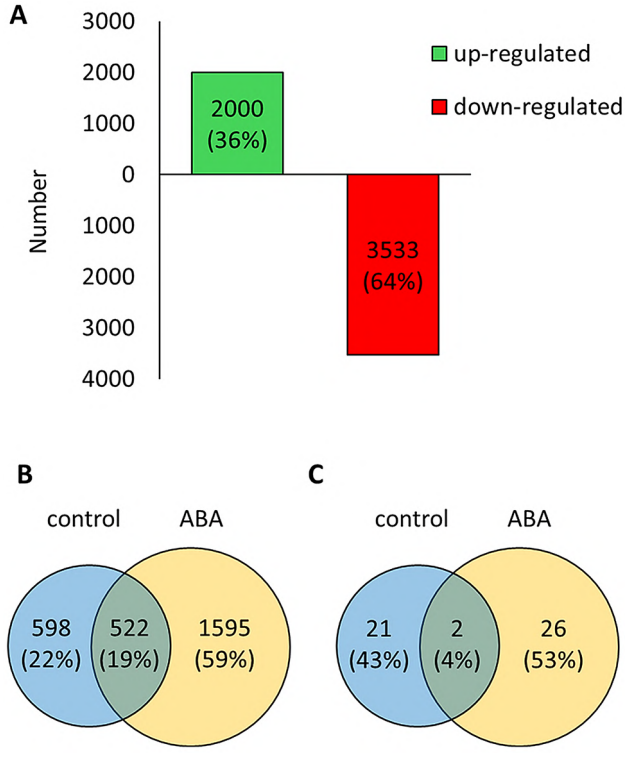


**Fig. 1** Differentially expressed genes (DEGs) in barley embryos germinating in the presence of 75 µM ABA compared to control conditions. (**A**) Number of upregulated and downregulated DEGs. (**B**) Venn diagram of poorly expressed genes (0–1 transcript per million [TPM]). (**C**) Venn diagram displaying the highly expressed genes (> 1000 TPM)

### Transcriptional regulation of ABA-treated germinating embryos

Given ABA effects on gene expression during germination, we focused on identifying the regulatory transcription factors. A total of 214 transcription factors (TFs) were identified. These belonged to 35 TF families, with the MYB family the most highly represented (35 genes) (Fig. 2; Additional file 2: Data S2). To determine whether the identified TFs could potentially regulate the expression of DEGs, we screened for binding sites within DEG promoter sequences. Of the 214 TFs, 23 had binding sites within 3,617 DEGs (Table 1; Additional file 3: Data S3). Several TFs specifically associated with the abscisic acid-activated signaling pathway (GO:0009738), including homologs of crucial ABA regulators such as AtABI3 (ABSCISIC ACID INSENSITIVE 3, BaRT2v18chr3HG161790), AtAREB3 (ABA-RESPONSIVE ELEMENT BINDING PROTEIN 3, BaRT2v18chr1HG033690), AtABF3 (ABSCISIC ACID-RESPONSIVE ELEMENT-BINDING FACTOR 3, BaRT-2v18chr3HG156370). ABI3 acts as the main regulator that controls seed dormancy and activates the ABA response. AREB3 and ABF3 further enhance the action of ABA by binding to ABRE (ABA-responsive elements) in target gene promoters, thereby intensifying the inhibitory effect on germination and effectively maintaining seeds in a dormant state [46–49]. This suggests a substantial regulatory influence of a limited number of TFs on the transcriptional response to ABA during seed germination.

### The role of ABA-regulated genes in germinating barley embryos

Gene Ontology (GO) analysis was used to highlight the biological processes associated with DEGs linked to ABA treatment (Fig. 3A; Additional file 4: Data S4; Additional file 5: Data S5). ABA downregulated genes were predominantly associated with processes such as cell wall organization or biogenesis (GO:0071554), phenylpropanoid biosynthetic process (GO:0009699), external encapsulating structure organization (GO:0045229),

99:1378425496

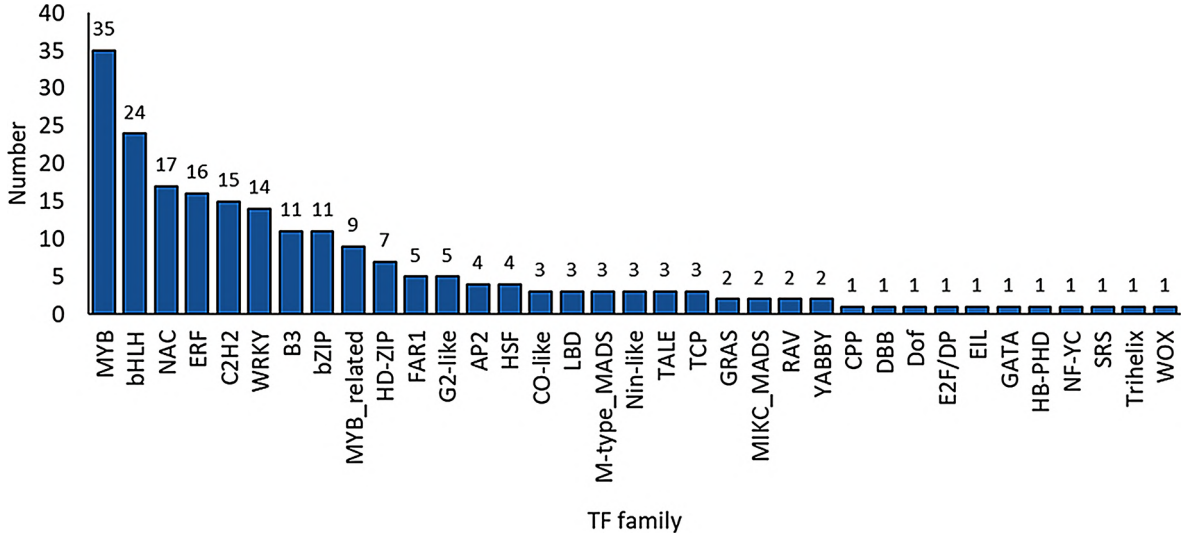


**Fig. 2** Transcription factors (TFs) in barley embryos germinating in the presence of 75 µM ABA compared to control conditions

**Table 1** Identified transcription factors (TF) with binding sites within DEGs

| BaRTv2 ID | TF family | Gene annotation | log2FC | Number of target genes | Gene Arabidopsis | Arabidopsis gene name |
|---|---|---|---|---|---|---|
| BaRT2v18chr2HG106120 | ERF | Ethylene-responsive transcription factor | 3.91 | 2271 | AT5G25190 | ESE3 |
| BaRT2v18chr1HG028050 | ERF | AP2/ERF domain-containing protein | 2.07 | 1900 | AT2G47520 | ERF71 |
| BaRT2v18chr3HG146510 | B3 | TF-B3 domain-containing protein | 2.67 | 1888 | AT1G28300 | LEC2 |
| BaRT2v18chr4HG208310 | C2H2 | C2H2-type domain-containing protein | 2.78 | 1609 | AT3G50700 | IDD2 |
| BaRT2v18chr1HG030570 | MIKC_MADS | PISTILLATA-like MADS-box transcription factor | -2.13 | 1530 | AT5G20240 | PI |
| BaRT2v18chr3HG161790 | B3 | Transcription factor VP-1 homologue | 2.80 | 1345 | AT3G24650 | ABI3 |
| BaRT2v18chr1HG046320 | bZIP | G-box binding factor | 2.78 | 1067 | AT4G01120 | GBF2 |
| BaRT2v18chr6HG319320 | HD-ZIP | Homeobox-leucine zipper family protein | -1.90 | 1062 | AT1G17920 | HDG12 |
| BaRT2v18chr5HG226660 | bZIP | BZIP transcription factor family | -4.58 | 1038 | AT3G54620 | BZIP25 |
| BaRT2v18chr3HG156370 | bZIP | ABA response element binding factor | 3.63 | 1021 | AT4G34000 | ABF3 |
| BaRT2v18chr1HG033690 | bZIP | BZIP transcription factor | 1.75 | 991 | AT3G56850 | AREB3 |
| BaRT2v18chr3HG119680 | RAV | AP2/B3 transcription factor family protein | -3.57 | 964 | AT3G25730 | EDF3 |
| BaRT2v18chr2HG077710 | bHLH | BHLH domain-containing protein | 2.06 | 931 | AT3G59060 | PIL6 |
| BaRT2v18chr3HG147640 | WRKY | WRKY transcription factor | -2.76 | 875 | AT1G29280 | WRKY65 |
| BaRT2v18chr1HG011410 | GATA | GATA-type domain-containing protein | -1.78 | 817 | AT3G06740 | GATA15 |
| BaRT2v18chr2HG059890 | G2-like | Two-component response regulator | 2.11 | 790 | AT3G25790 | HHO1 |
| BaRT2v18chr2HG092760 | TCP | TCP transcription factor | -3.42 | 785 | AT5G23280 | TCP7 |
| BaRT2v18chr2HG058120 | NAC | NAC domain-containing protein | -1.91 | 765 | AT5G61430 | NAC100 |
| BaRT2v18chr1HG014170 | MYB | HTH myb-type domain-containing protein | -4.62 | 691 | AT5G11510 | MYB3R-4 |
| BaRT2v18chr3HG150290 | TCP | TCP family transcription factor containing protein | -1.99 | 584 | AT5G60970 | TCP5 |
| BaRT2v18chr4HG205400 | AP2 | AP2 domain containing protein | 1.95 | 578 | AT4G37750 | ANT |
| BaRT2v18chr1HG030650 | NAC | NAC domain-containing protein | 3.19 | 528 | AT1G01720 | ATAF1 |
| BaRT2v18chr6HG317740 | E2F/DP | E2F transcription factor | -2.38 | 418 | AT3G01330 | DEL3 |

melatonin metabolism (GO:0030186), photosynthesis light reaction (GO:0019684), response to oxidative stress (GO:0006979), nucleosome assembly (GO:0006334), lignin biosynthetic process (GO:0009809), generation of precursor metabolites and energy (GO:0006091) and chromatin remodeling (GO:0006338). In contrast, upregulated genes were primarily linked to responses to abscisic acid (GO:0009737), alcohol (GO:0097305), oxygen-containing compounds (GO:1901700), cold acclimation (GO:0009631), response to salt (GO:1902074),

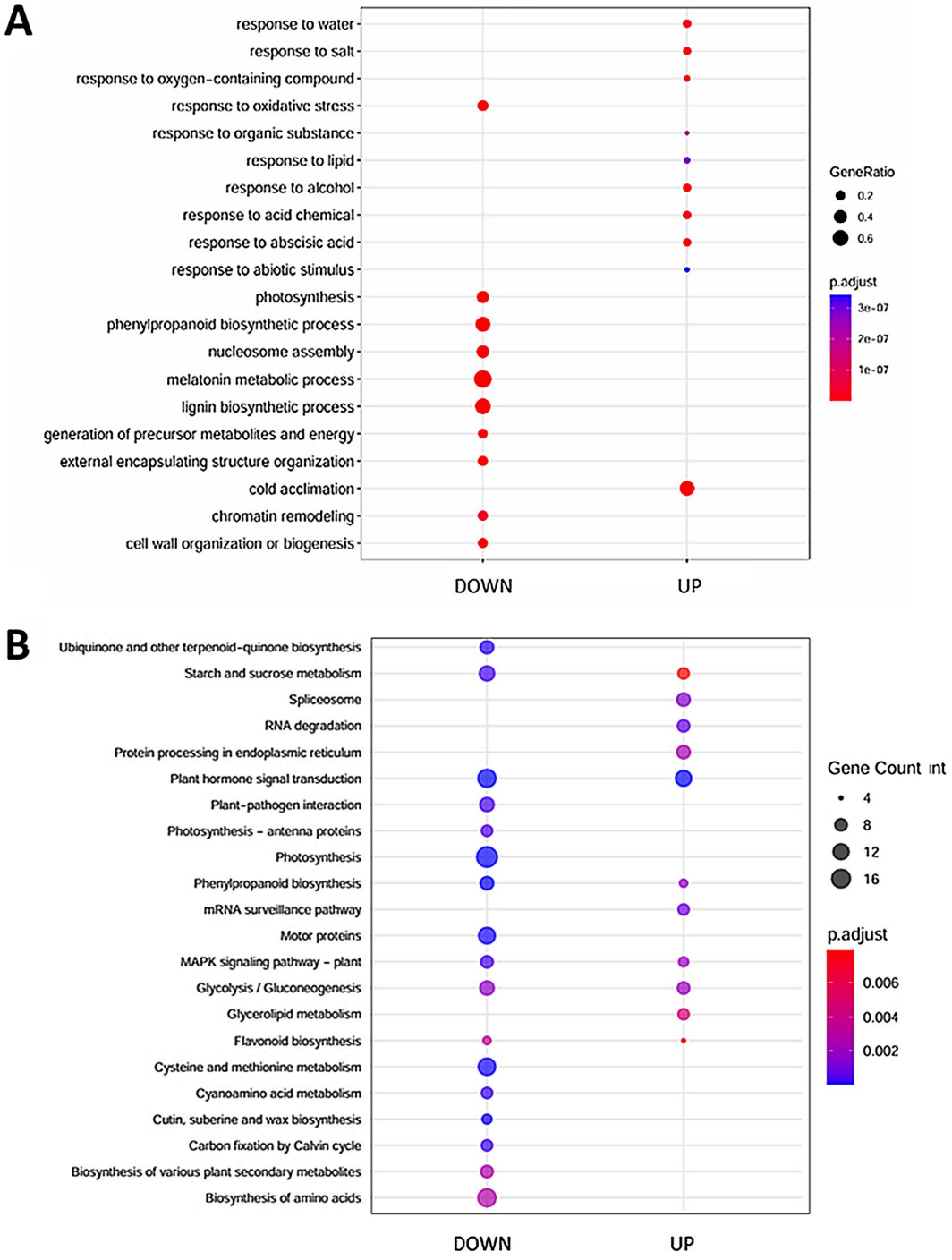


**Fig. 3** GO enrichment and KEGG analysis of up- and downregulated genes differentially expressed genes (DEGs) in barley embryos germinating in the presence of 75 µM ABA compared to control conditions. (**A**) GO analysis. (**B**) KEGG pathway analysis. GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes)

response to water (GO:0009415), response to acid chemical (GO:0001101), response to organic substance (GO:0010033), response to lipid (GO:0033993) and response to abiotic stimulus (GO:0009628). Taken together, ABA treatment broadly inhibits metabolic processes and structural organization in the embryo, and may simultaneously enhance its adaptive responses to adverse environmental conditions. Moreover, we found that 44 DEGs were involved in responses to ABA (GO:0009737) (Additional file 6: Data S6). These included genes encoding seven TFs, including three ABA-related TFs (AtABI3, AtAREB3, AtABF3), key components of the

ABA signaling pathway, such as four SNF1-RELATED PROTEIN KINASE 2 (SnRK2s), and five PROTEIN PHOSPHATASE 2 C (PP2Cs), ten LEA (LATE EMBRYOGENESIS ABUNDANT) proteins, particularly dehydrins, as well as one ABA transporter.

To further explore the roles of the DEGs, we utilized the KEGG online database (Fig. 3B; Additional file 7: Data S7). Out of the 1,772 upregulated and only 396 (22%) and 938 (30%) downregulated DEGs were assigned to KEGG pathways. Downregulated DEGs were predominantly involved in cyanoamino acid metabolism (ko00460), carbon fixation by Calvin cycle (ko00710), glycolysis/gluconeogenesis (ko00010), motor proteins (ko04814), cysteine and methionine metabolism (ko00270), cutin, suberine, and wax biosynthesis (ko00073), ubiquinone and other terpenoid-quinone biosynthesis (ko00130), photosynthesis-antenna proteins (ko00196), photosynthesis (ko00195), phenylpropanoid biosynthesis (ko00940), MAPK signaling pathway—plant (ko04016), plant-pathogen interaction (ko04626), starch and sucrose metabolism (ko00500), plant hormone signal transduction (ko04075), flavonoid biosynthesis (ko00941), biosynthesis of amino acids (ko01230), and biosynthesis of various plant secondary metabolites (ko00999). The upregulated DEGs were involved in protein processing in the endoplasmic reticulum (ko04141), RNA degradation (ko03018), glycolysis/gluconeogenesis (ko00010), MAPK signaling pathway (ko04016), phenylpropanoid biosynthesis (ko00940), spliceosome (ko03040), mRNA surveillance pathway (ko03015), plant hormone signal transduction (ko04075), starch and sucrose metabolism (ko00500), flavonoid biosynthesis (ko00941), and glycerolipid metabolism (ko00561).

### Phytohormonal control of germinating barley embryos in response to ABA

Given the well-established role of ABA, with other phytohormones, in regulating seed germination, the 'plant hormone signal transduction' pathway has become a key focus of our analysis [1, 50, 51]. The KEGG pathway map illustrates the various plant phytohormone pathways that are differentially regulated in germinating embryos in response to ABA treatment (Fig. 4). Increased activity was observed within the ABA signaling pathway, where elements such as the PYRABACTIN RESISTANCE 1-LIKE (PYR/PYL) receptor family, phosphatases PP2Cs, kinases SnRK2s, and AREB/ABFs transcription factors were identified (Table 2; Additional file 8: Data S8).

Increased ABA biosynthesis was shown by the upregulation of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*) and two genes annotated as *BETA-CAROTENE 3-HYDROXYLASE* (Table 3; Additional file 9: Data S9). Genes from other phytohormonal pathways, including auxin, jasmonic acid (JA), gibberellin (GA), ethylene (ET), cytokinin (CTK), and salicylic acid (SA), were also targeted. In addition, genes involved in calcium signaling pathways, which are crucial for cell division and growth processes, were mapped, along with components of phosphorylation cascades and plasma membrane transport systems that contribute to enhanced growth responses (Table 2; Fig. 4; Additional file 8: Data S8). The KEGG pathway map analysis also showed DEGs within several key pathways, including the biosynthesis of JA, BR, diterpenoids (including the biosynthesis of GA), ET biosynthesis, zeatin, and tryptophan metabolism pathway related to the production of indole-3-acetic acid (IAA) (Table 3; Additional file 9: Data S9). These results suggest a broad network of phytohormonal crosstalk triggered by ABA within the embryo.

### Comparative analysis of transcriptomics and metabolomics in germinating barley embryos in response to ABA

To complement our transcriptome analysis, we investigated the metabolomic changes in barley embryos under ABA treatment to gain a broader understanding of the molecular response. PLS-DA showed that ABA treatment resulted in a clear metabolic shift in the embryo (Fig. 5A). The top loading vectors for the metabolomic data were related to phytohormonal pathways and 25 showed decreased accumulation, whereas 7 showed increased levels after ABA treatment (Additional file 10: Data S10). Within this phytohormonal groups, ABA pathways were prominent, and it appeared that ABA treatment initiated further endogenous ABA production as shown by statistically significant increases in violaxanthin (p-value = 0.02; FC = 1.76) and β-carotene (p-value = 0.04; FC = 1.90) (Fig. 5B). When relating these to ABA associated DEGs, 20 were upregulated and 15 were downregulated (Table 4).

The ABA catabolic pathway was also altered, supported by reduced levels of the major catabolic metabolite of ABA-phaseic acid (PA). This is consistent with the increased expression of genes encoding key enzymes in the ABA biosynthesis pathway, *9-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*), and *BETA-CAROTENOID HYDROXYLASES*. In addition, ABA signaling-related genes, such as *PYR/PYL*, *PP2Cs*, *SnRK2s* and *AREB/ABFs* also showed altered expression (Table 2; Additional file 8: Data S8; Table 3; Additional file 9: Data S9). In addition to ABA, other phytohormones also play a significant role in differentiating responses in both metabolomic and transcriptomic data. Metabolites related to gibberellins (GA12, GA12-aldehyde, GA15, GA17, GA24, and GA44), brassinosteroids (brassinolide, castasterone, deoxocastasterone, and campesterol), salicylic acid, jasmonates (OPDA, linolenic acid, and HPTOE), and strigolactones (sorgolactone) were major sources of variation. Considering DEGs, these included

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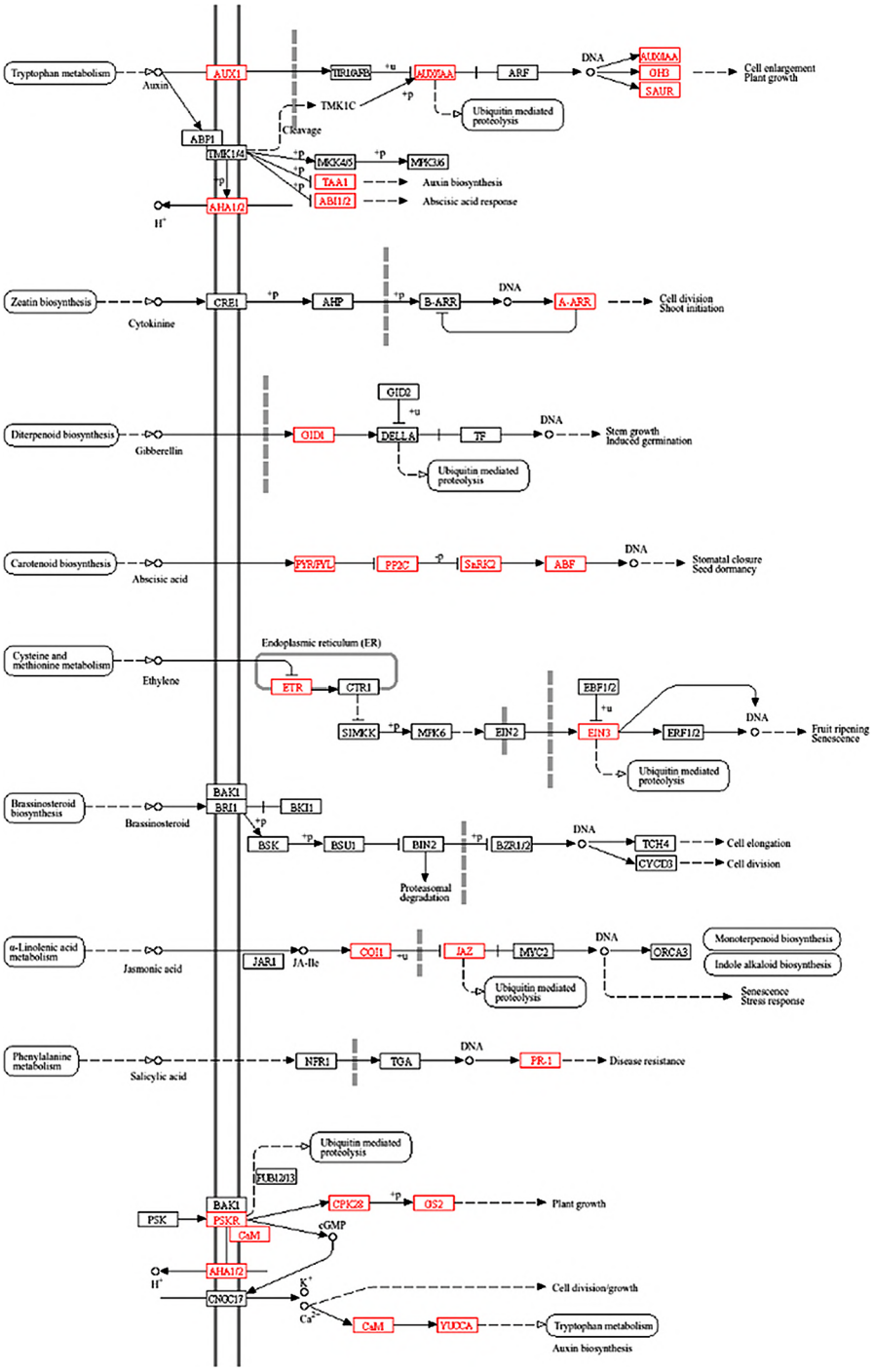


**Fig. 4** Visualization of plant hormone signal transduction KEGG pathway. Red colour representing upregulation and green colour representing downregulation

transcripts from the GA, SA, JA, CTK, and auxin pathways suggests the involvement of these phytohormones in the regulation of seed germination in response to ABA (Table 4; Additional file 10: Data S10). Integration and comparison of the phytohormone associated DEGs and metabolites suggested a high degree of correlation (0.99) (Fig. 6). The key associations are illustrated using circos plot using a correlation coefficient cutoff of > 0.9 to indicate both positive and negative associations (Fig. 7A; Additional file 11: Data S11). The gene correlations with ABA were highlighted in a network centered on ABA (Fig. 7B). Unsurprisingly, ABA levels were positively correlated with the key signaling components *SnRK2*, *ABF* and *PP2C*. ET (*ETR/ERS*, *EIN3*), jasmonate (*COI1*) and

**Table 2** DEGs in germinating barley embryos after ABA treatment linked to plant hormone signal transduction (KEGG)

| KEGG identifier | KEGG Name | KEGG Symbol | BaRTv2 ID |
|---|---|---|---|
| K01535 | H+-transporting ATPase | AHA1/2 (PMA1/PMA2) | BaRT2v18chr4HG177050 |
| K01915 | glutamine synthetase | GS (glnA, GLUL) | BaRT2v18chr2HG105870; BaRT2v18chr4HG178760 |
| K11816 | indole-3-pyruvate monooxygenase | YUCCA | BaRT2v18chr5HG232860; BaRT2v18chr2HG108970; BaRT2v18chr3HG147750 |
| K13412 | calcium-dependent protein kinase | CPK | BaRT2v18chr5HG269710; BaRT2v18chr3HG153220; BaRT2v-18chr4HG177010; BaRT2v18chr5HG238490; BaRT2v18chr5HG269680; BaRT2v18chr5HG256940 |
| K13463 | coronatine-insensitive protein 1 | COI1 | BaRT2v18chr1HG036610 |
| K14432 | ABA responsive element binding factor | AREB/ABF | BaRT2v18chr3HG156370; BaRT2v18chr1HG033690 |
| K14488 | SAUR family protein | SAUR | BaRT2v18chr7HG378910; BaRT2v18chr7HG374000; BaRT2v-18chr6HG319690; BaRT2v18chr6HG292090; BaRT2v18chr2HG105250; BaRT2v18chr2HG105200; BaRT2v18chr2HG105230 |
| K14493 | gibberellin receptor GID1 | GID1 | BaRT2v18chr1HG028980 |
| K14497 | protein phosphatase 2 C | PP2C | BaRT2v18chr3HG142490; BaRT2v18chr1HG046520; BaRT2v-18chr2HG049520; BaRT2v18chr3HG157400; BaRT2v18chr3HG138810 |
| K14498 | serine/threonine-protein kinase SRK2 | SnRK2 | BaRT2v18chr1HG037480; BaRT2v18chr1HG026070; BaRT2v18chr4HG182300 |
| K14509 | ethylene receptor | ETR | BaRT2v18chr6HG314730 |
| K14514 | ethylene-insensitive protein 3 | EIN3 | BaRT2v18chr2HG086440 |
| K02183 | calmodulin | CaM | BaRT2v18chr1HG033520; BaRT2v18chr5HG238570 |
| K13449 | pathogenesis-related protein 1 | PR1 | BaRT2v18chr7HG337030; BaRT2v18chr5HG270910; BaRT2v-18chr7HG342320; BaRT2v18chr5HG270890; BaRT2v18chr7HG346350; BaRT2v18chr5HG244050 |
| K13464 | jasmonate ZIM domain-containing protein | JAZ | BaRT2v18chr2HG106520; BaRT2v18chr2HG082260 |
| K13946 | auxin influx carrier (AUX1 LAX family) | AUX1/LAX | BaRT2v18chr4HG185730; BaRT2v18chr5HG271040; BaRT2v-18chr5HG226700; BaRT2v18chr1HG013580; BaRT2v18chr5HG264940; BaRT2v18chr5HG226680; BaRT2v18chr3HG148390; BaRT2v-18chr3HG124860; BaRT2v18chr7HG366910; BaRT2v18chr7HG339240 |
| K14487 | auxin responsive GH3 gene family | GH3 | BaRT2v18chr3HG152100; BaRT2v18chr2HG091440; BaRT2v18chr2HG059720 |
| K14492 | two-component response regulator ARR-A family | A-ARR | BaRT2v18chr2HG111060; BaRT2v18chr5HG237800; BaRT2v-18chr2HG085110; BaRT2v18chr2HG092540 |
| K14496 | abscisic acid receptor PYR/PYL family | PYL | BaRT2v18chr1HG034770 |
| K16903 | L-tryptophan-pyruvate aminotransferase | TAA1 | BaRT2v18chr3HG123080 |
| K27625 | phytosulfokine receptor 1 | PSKR1 | BaRT2v18chr6HG307580 |

gibberellin (*GID1*) associated gene expression also positively correlated with ABA. Also positively correlated with ABA were genes linked to lipid-associated events (*DGKA*, *DGAT1*, *DPP1*, *DGAT2*, *GPAT*). A negative correlation was seen with most auxin-associated genes (*SAUR.a*, *YUCCA.a*, *TAA1*, *AUX1/LAX*) and also with *PR1*, which is a marker for salicylate effects.

### Insights from bulk RNA-seq and spatial transcriptomics into ABA-dependent genetic regulation of seed development and germination

Next, we compared the transcriptomic profiles of ABA-treated barley embryos with those of developing seeds. For this purpose, we selected 3,621 DEGs with HORVU. MOREX identifiers from our RNA-seq data. This dataset was compared to the DEGs identified by Kovacik et al. (2024), where 15,627 DEGs were detected in the embryo, 20,618 DEGs in the endosperm, and 12,638 DEGs in the seed maternal tissue (SMT) during seed development [44]. Comparative analysis of these datasets revealed common genes between ABA-treated germinating embryos and individually developing seed tissues: 2,035 genes, 2,219 genes, and 1,813 genes in the embryo, endosperm and SMT, respectively. In addition, ABA treatment-dependent DEGs were identified in each of these tissues: 1,586 genes in the embryo, 1,402 genes in the endosperm, and 1,898 genes in the SMT (Fig. 8A, B, C). Next, focusing on the embryo tissue, we analyzed

**Table 3** DEGs in germinating barley embryos after ABA treatment linked to plant hormone biosynthesis pathways (KEGG)

| KEGG pathway map name | KEGG pathway map ID | KEGG identifier | KEGG Symbol | BaRTv2 ID |
|---|---|---|---|---|
| Jasmonic acid biosynthesis | map00592 | K00454 | LOX2S | BaRT2v18chr5HG221560; BaRT2v18chr6HG282910 |
| | | K05894 | OPR | BaRT2v18chr7HG343660; BaRT2v18chr7HG343690; BaRT2v18chr7HG330350; BaRT2v18chrUnG390320; BaRT2v18chr7HG343680; BaRT2v18chr7HG385100 |
| Brassinosteroid biosynthesis | map00905 | K15639 | CYP734A1, BAS1 | BaRT2v18chr6HG299590; BaRT2v18chr3HG126970; BaRT2v18chr2HG062140 |
| | | K20623 | CYP92A6 | BaRT2v18chr5HG247530; BaRT2v18chr7HG347310 |
| Diterpenoid biosynthesis | map00904 | K04122 | GA3 | BaRT2v18chr7HG368050 |
| | | K04125 | GA2ox | BaRT2v18chr2HG092430 |
| | | K05282 | GA20ox | BaRT2v18chr1HG030670 |
| | | K16085 | CYP99A2_3 | BaRT2v18chr2HG052040; BaRT2v18chr2HG055770 |
| Ethylene biosynthesis | map00270 | K00789 | metK, MAT | BaRT2v18chr6HG310160; BaRT2v18chr6HG310120 |
| | | K01762 | ACS | BaRT2v18chr3HG124710 |
| | | K05933 | E1.14.17.4 | BaRT2v18chr5HG250670; BaRT2v18chr4HG184710; BaRT2v18chr6HG319390 |
| | | K20772 | ACS1_2_6 | BaRT2v18chr2HG095020 |
| Abscisic acid biosynthesis | map00906 | K09840 | NCED | BaRT2v18chr5HG223780 |
| | | K15746 | beta-carotene 3-hydroxylase | BaRT2v18chr2HG094980; BaRT2v18chr4HG215920 |
| Zeatin biosynthesis | map00908 | K00279 | cytokinin dehydrogenase | BaRT2v18chr1HG019230; BaRT2v18chr5HG246980 |
| | | K13495 | cis-zeatin O-glucosyltransferase | BaRT2v18chr2HG096460; BaRT2v18chr2HG096430 |
| Tryptophan metabolism | map00380 | K01426 | E3.5.1.4; amidase | BaRT2v18chr2HG051690; BaRT2v18chr2HG051700 |
| | | K11816 | YUCCA | BaRT2v18chr5HG232860; BaRT2v18chr2HG108970; BaRT2v18chr3HG147750 |
| | | K16903 | TAA1 | BaRT2v18chr3HG123080 |
| | | K22450 | SNAT | BaRT2v18chr7HG363550 |
| | | K22588 | ASMT | BaRT2v18chr1HG001560 |

GO-BP functions for 1,586 ABA treatment-dependent DEGs (Additional file 12: Data S12). The most enriched GO biological processes were cell wall organization and cell structure modification, such as cell wall organization or biogenesis (GO:0071554), external encapsulating structure organization (GO:0045229), hemicellulose metabolic process (GO:0010410), polysaccharide metabolic process (GO:0005976). The processes related to the response to stress were also altered: response to oxidative stress (GO:0006979), phenylpropanoid biosynthetic process (GO:0009699), oligopeptide transport and metabolism (GO:0006857), nitrate transmembrane transport (GO:0015706), regulation of enzymatic activity (GO:0080163), and negative regulation of hydrolase activity (GO:0051346) (Fig. 8D; Additional file 13: Data S13; Additional file 14: Data S14). Among the DEGs identified, 2,035 overlapped with the DEGs expressed in the embryo during seed development, as reported by Kovacik et al. (2024) [44] (Additional file 15: Data S15). This overlap highlights the conserved processes regulated by ABA in stress responses, as well as during seed development. GO enrichment analysis revealed that the functions of these genes are linked to cell movement and division (e.g., microtubule-based movement (GO:0007018), mitotic cell cycle phase transition (GO:0044772). Furthermore, GO-BP processes related to stress and abiotic factor responses were enriched, such as response to abscisic acid (GO:0009737), response to water deprivation (GO:0009414), cold acclimation (GO:0009631), and response to salt (GO:1902074), among others (Fig. 8E; Additional file 16: Data S16; Additional file 17: Data S17). The response to ABA (GO:0009737) represents a common and critical process shared between ABA treatment and embryo development.

To extend our analysis beyond bulk RNA-seq and capture spatial gene expression patterns, we performed Visium spatial transcriptomics (10× Genomics) to gain deeper insights into tissue-specific ABA-responsive gene expression. We were able to precisely localize gene expression across six germinating embryo tissues in response to ABA: coleoptile, cotyledon, mesocotyl, plumule, scutellum, and radicle (Fig. 9A). Among 1,586 ABA treatment-dependent DEGs identified in our bulk RNA-seq experiment, we assigned tissue-specific expression to 49 DEGs (Fig. 9B). 30 of these genes were expressed in the coleoptile, 20 in the scutellum, 16 in the radicle, 12 in the mesocotyl, 9 in the plumule, and 2 in the cotyledon. The coleoptile tissue expressed the largest number of tissue-specific genes, accounting for 14 DEGs. Single tissue expression was also observed for 8 DEGs in the radicle,

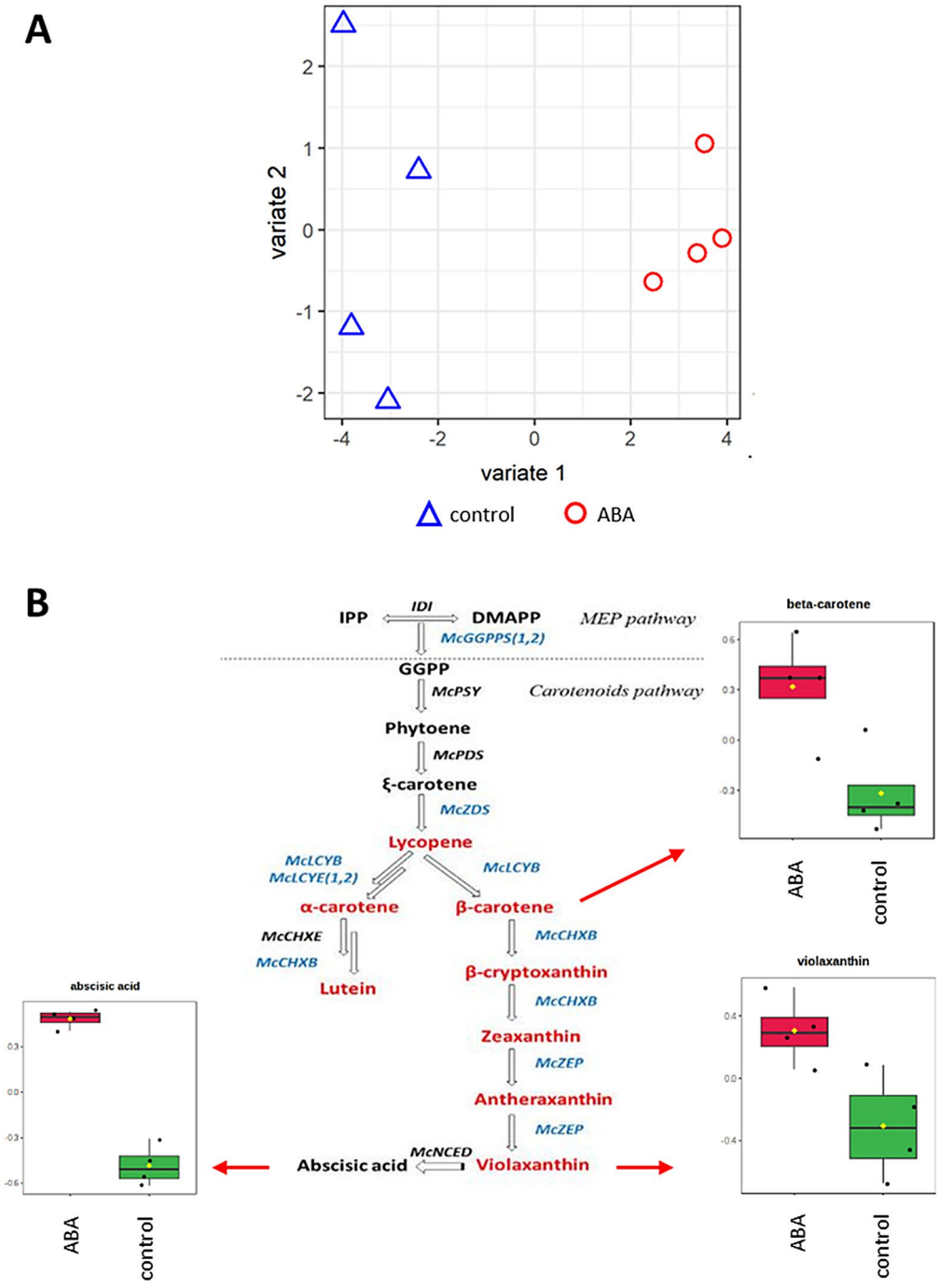


**Fig. 5** (**A**) Partial Least Squares Discriminant Analysis (PLS-DA) of metabolomic profiles obtained from barley embryos germinating in the presence of 75 µM ABA and in the control conditions. Control samples are represented by blue triangles and ABA samples by red circles. (**B**) Quantitative analysis of key metabolites in the carotenoid biosynthesis pathway in barley embryos germinating with 75 µM ABA compared to the control conditions. Box plots show normalized concentrations of identified metabolites

**Table 4** Phytohormone-related DEGs in the transcriptomic block in germinating barley embryos after ABA treatment

| BaRTv2 ID | Gene annotation | log2FC | KEGG ID | KEGG symbol |
|---|---|---|---|---|
| BaRT2v18chr6HG307580 | Phytosulfokine receptor 1 | -2.18 | K27625 | PSKR1 |
| BaRT2v18chr1HG018060 | Esterase/lipase/thioesterase-like protein | 1.51 | K27538 | PES |
| BaRT2v18chr7HG349770 | Diacylglycerol O-acyltransferase 2 | 1.86 | K22848 | DGAT2 |
| BaRT2v18chr3HG119330 | Lipid phosphate phosphatase-like protein | 2.01 | K18693 | DPP1, DPPL, PLPP4_5 |
| BaRT2v18chr3HG123080 | Tryptophan aminotransferase | -1.83 | K16903 | TAA1 |
| BaRT2v18chr2HG086440 | Ethylene-insensitive 3 | 5.44 | K14514 | EIN3 |
| BaRT2v18chr6HG314730 | Ethylene receptor | 3.20 | K14509 | ETR |
| BaRT2v18chr1HG037480 | SnRK2 serine threonine protein kinase | -2.91 | K14498 | SnRK2 |
| BaRT2v18chr4HG182300 | SnRK2 serine threonine protein kinase | 1.76 | K14498 | SnRK2 |
| BaRT2v18chr3HG138810 | Protein-serine/threonine phosphatase | 1.91 | K14497 | PP2C |
| BaRT2v18chr1HG034770 | Abscisic acid receptor | -2.46 | K14496 | PYL |
| BaRT2v18chr1HG028980 | Gibberellin receptor GID1a | 2.05 | K14493 | GID1 |
| BaRT2v18chr2HG092540 | Response regulatory domain-containing protein | -2.80 | K14492 | A-ARR |
| BaRT2v18chr2HG105230 | Auxin responsive SAUR protein | 1.55 | K14488 | SAUR |
| BaRT2v18chr6HG292090 | Auxin responsive SAUR protein | -3.60 | K14488 | SAUR |
| BaRT2v18chr2HG059720 | GH3 family protein | -2.45 | K14487 | GH3 |
| BaRT2v18chr7HG339240 | Auxin-responsive protein | -4.03 | K14484 | AUX/IAA |
| BaRT2v18chr1HG033690 | BZIP transcription factor | 1.75 | K14432 | AREB/ABF |
| BaRT2v18chr4HG185730 | Auxin influx transporter | -3.72 | K13946 | AUX1/LAX |
| BaRT2v18chr5HG276890 | Putative glycerol-3-phosphate 1-O-acyltransferase | 3.59 | K13508 | GPAT |
| BaRT2v18chr2HG082260 | Tify domain-containing protein | -3.50 | K13464 | JAZ |
| BaRT2v18chr1HG036610 | Coronatine insensitive protein 1 | 1.61 | K13463 | COI1 |
| BaRT2v18chr5HG244050 | Pathogenesis-related protein class 1 | -8.06 | K13449 | PR1 |
| BaRT2v18chr6HG299270 | Respiratory burst oxidase | 1.78 | K13447 | RBOH |
| BaRT2v18chr5HG256940 | Calcium-dependent protein kinase | 2.34 | K13412 | CPK |
| BaRT2v18chr5HG269680 | Calcium-dependent protein kinase | -9.00 | K13412 | CPK |
| BaRT2v18chr2HG108970 | Flavin-containing monooxygenase | -4.96 | K11816 | YUCCA |
| BaRT2v18chr3HG147750 | Flavin-containing monooxygenase | 1.80 | K11816 | YUCCA |
| BaRT2v18chr7HG368430 | O-acyltransferase | 2.39 | K11155 | DGAT1 |
| BaRT2v18chr2HG052310 | Heat shock protein | 3.42 | K04079 | HSP90A, htpG |
| BaRT2v18chr5HG238570 | Calmodulin protein | -4.70 | K02183 | CaM |
| BaRT2v18chr2HG105870 | Glutamine synthetase | -2.14 | K01915 | GS (glnA, GLUL) |
| BaRT2v18chr4HG178760 | Glutamine synthetase | 2.03 | K01915 | GS (glnA, GLUL) |
| BaRT2v18chr4HG177050 | Plasma membrane ATPase | 3.55 | K01535 | AHA1/2 (PMA1/PMA2) |
| BaRT2v18chr1HG027420 | Diacylglycerol kinase | 2.92 | K00901 | dgkA, DGK |

7 DEGs in the scutellum, and 2 DEGs in the mesocotyl. Additionally, 18 DEGs were present in more than one tissue possibly indicated ABA-dependent genes have common functions in different embryonic regions. A comparison of bulk RNA-seq and spatial transcriptomics results revealed a high agreement in the overall gene expression pattern, indicating a consistency of results obtained using both technologies (Table 5).

## Discussion

In our study, ABA treatment was shown to reduce the expression of a significant number of DEGs in germinating barley embryos. A similar effect was observed in *Arabidopsis thaliana* embryos and germinating wheat embryos, where ABA treatment also led to strong repression of the expression of most genes by 62% and 59%, respectively [52, 53]. These results suggest that ABA acts as a key gene-repressive regulator in embryos. The impact of ABA treatment on transcriptional regulation is further reflected by its influence on the expression of specific transcription factor families. The largest number of transcription factors was from the MYB family in germinating embryos treated with ABA. Plant MYB proteins are distinguished by the highly conserved MYB domain responsible for DNA binding and are involved in a broad spectrum of biological processes such as plant development, secondary metabolism, hormonal signal transduction, disease resistance, and tolerance to abiotic stresses [54]. MYB TFs also play a vital role in the regulation of seed germination. For example, MYB70 inhibits germination in an ABA-dependent manner by interacting with ABI5 [55]. MYB96 cooperates with ABI4 to control lipid mobilization in embryos [56]. In addition, MYB94 and MYB330 modulate the germination process by affecting

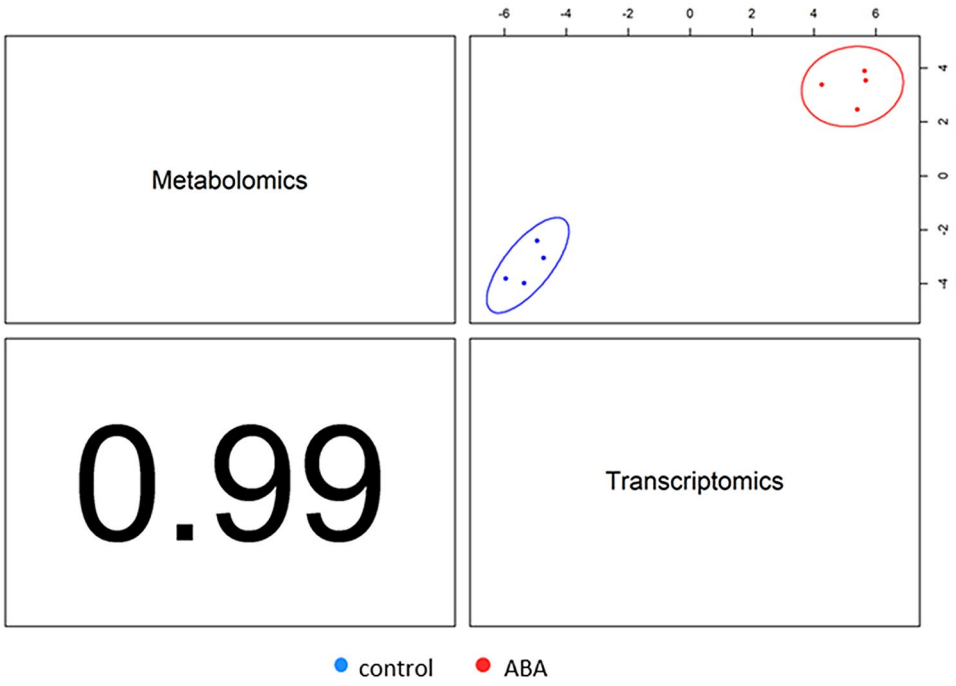


**Fig. 6** Correlation between metabolomic and transcriptomic data from barley embryos germinating in the presence of 75 µM ABA and in the control conditions. Control samples are shown in blue, and ABA samples in red

ABA-dependent signaling pathways [57, 58]. These observations highlight the role of MYB TFs in transcriptional regulation of the response of germinating embryos to ABA-related hormonal signals. Moreover, in our study, we identified 23 TFs with binding sites within DEGs, including bZIP transcription factors such as AtABI3, AtAREB3 and AtABF3, which encode key ABA response regulators. These TFs transduce ABA signals by binding to specific ABA-responsive elements (ABREs) in promoter regions. This coordinated interaction fine-tunes seed responses under stress conditions, ensuring the precise regulation of ABA-responsive genes involved in the germination process [59–62]. GO and KEGG analyses revealed that ABA treatment suppressed metabolic and biosynthetic processes, such as photosynthesis and lignin biosynthesis, while simultaneously activating pathways related to stress response, phytohormone signaling, and environmental adaptation. This suggests that ABA redirects the plant's physiological priorities from growth and energy production to enhance its ability to cope with stressful conditions.

Our integrative transcriptomic and metabolomic approach revealed a strong interplay between ABA treatment and a broader phytohormonal network. A three-fold increase in ABA accumulation in germinating embryos in the presence of this phytohormone was associated with an increase in the number of key compounds in the ABA biosynthetic pathway, such as violaxanthin and beta-carotene, with the increased expression of ABA biosynthesis genes, and a significant decrease in phasic acid, which is the main catabolic metabolite of ABA. This was also associated with changes in the ABA signaling pathway, with increases in the expression of *SnRK2s* kinase genes (*BaRT2v18chr1HG026070*, *BaRT2v18chr4HG182300*), which activate ABF/AREB transcription factors (BaRT-2v18chr3HG156370, BaRT2v18chr1HG033690), triggering adaptation to stress conditions. Simultaneously, there was a decrease in the expression of one *SnRK2* kinase (*BaRT2v18chr1HG037480*), which is linked to a modulation of the intensity of ABA signaling. In our study, the expression of the *PYL* gene (*BaRT2v18chr1HG034770*) was reduced, while the expression of *PP2C* phosphatases (*BaRT2v18chr3HG142490*, *BaRT2v18chr1HG046520*, *BaRT2v18chr2HG049520*, *BaRT2v18chr3HG157400*, and *BaRT2v18chr3HG138810*) was increased. It is known that the activation of the core ABA signaling pathway in response to ABA starts with the binding of ABA to PYR/PYL receptors, which blocks the action of clade A PP2C phosphatases and initiates the response to ABA [63–65]. However, the expression of genes encoding *PYR/PYL* receptors may be reduced and the expression of genes encoding *PP2Cs* may be increased to prevent the excessive response to ABA-induced stress [66, 67]. Our results align with such a modulatory feedback mechanism, suggesting a careful tuning of ABA signaling under stress conditions.

Given the known antagonistic roles of ABA and gibberellin (GA) in seed germination, GA metabolism was also

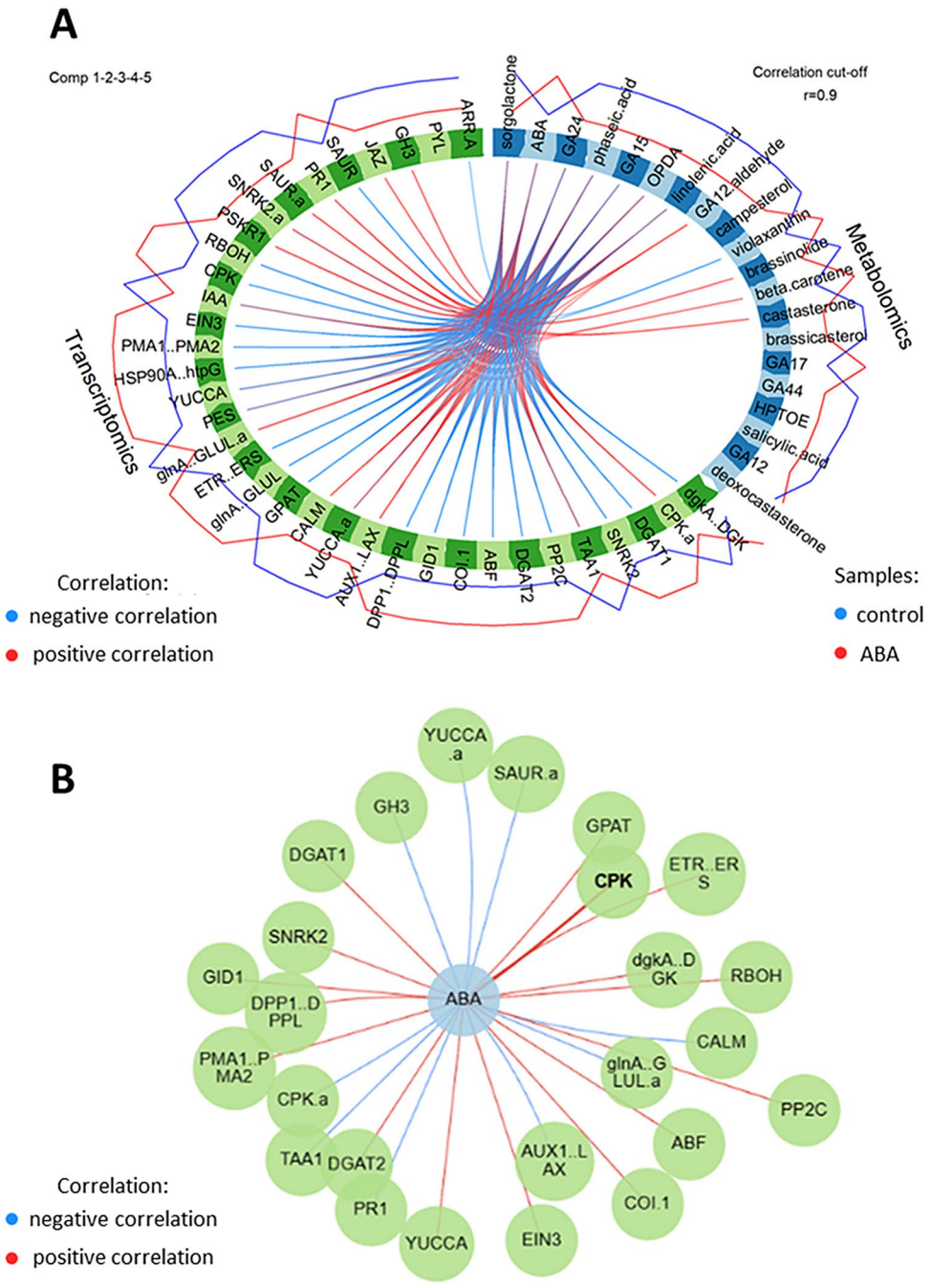


**Fig. 7** Interactions between hormone networks. (**A**) Circos plot showing correlations between key transcriptomic and metabolomic parameters in samples obtained from barley embryos germinating in the presence of 75 µM ABA and in the control conditions. Red lines within the circos plot indicate positive and blue lines represent negative correlations. The red line outside the circus plot shows levels in ABA-treated samples, and the blue line represents levels in control samples. Correlation cut-off $r = 0.9$. (**B**) Abscisic acid (blue circle) interactions with genes (green circles). *ARR-A* (*BaRT2v18chr2HG092540*), *PYL* (*BaRT2v18chr1HG034770*), *GH3* (*BaRT2v18chr2HG059720*), *JAZ* (*BaRT2v18chr2HG082260*), *SAUR* (*BaRT2v18chr2HG105230*), *PR1* (*BaRT2v18chr5HG244050*), *SAUR.a* (*BaRT2v18chr6HG292090*), *SNRK2.a* (*BaRT2v18chr1HG037480*), *PSKR1* (*BaRT2v18chr6HG307580*), *RBOH* (*BaRT2v18chr6HG299270*), *CPK* (*BaRT2v18chr5HG256940*), *IAA* (*BaRT2v18chr7HG339240*), *EIN3* (*BaRT2v18chr2HG086440*), *PMA1.PMA2* (*BaRT2v18chr4HG177050*), *HSP90A.htpG* (*BaRT2v18chr2HG052310*), *YUCCA* (*BaRT2v18chr3HG147750*), *PES* (*BaRT2v18chr1HG018060*), *glnA.GLUL.a* (*BaRT2v18chr2HG105870*), *ETR.ERS* (*BaRT2v18chr6HG314730*), *glnA.GLUL* (*BaRT2v18chr4HG178760*), *GPAT* (*BaRT2v18chr5HG276890*), *CALM* (*BaRT2v18chr5HG238570*), *YUCCA.a* (*BaRT2v18chr2HG108970*), *AUX1.LAX* (*BaRT2v18chr4HG185730*), *DPP1.DPPL* (*BaRT2v18chr3HG119330*), *GID1* (*BaRT2v18chr1HG028980*), *COI.1* (*BaRT2v18chr1HG036610*), *ABF* (*BaRT2v18chr1HG033690*), *DGAT2* (*BaRT2v18chr7HG349770*), *PP2C* (*BaRT2v18chr3HG138810*), *TAA1* (*BaRT2v18chr3HG123080*), *SNRK2* (*BaRT2v18chr4HG182300*), *DGAT1* (*BaRT2v18chr7HG368430*), *CPK.a* (*BaRT2v18chr5HG269680*), *dgkA.DGK* (*BaRT2v18chr1HG027420*)

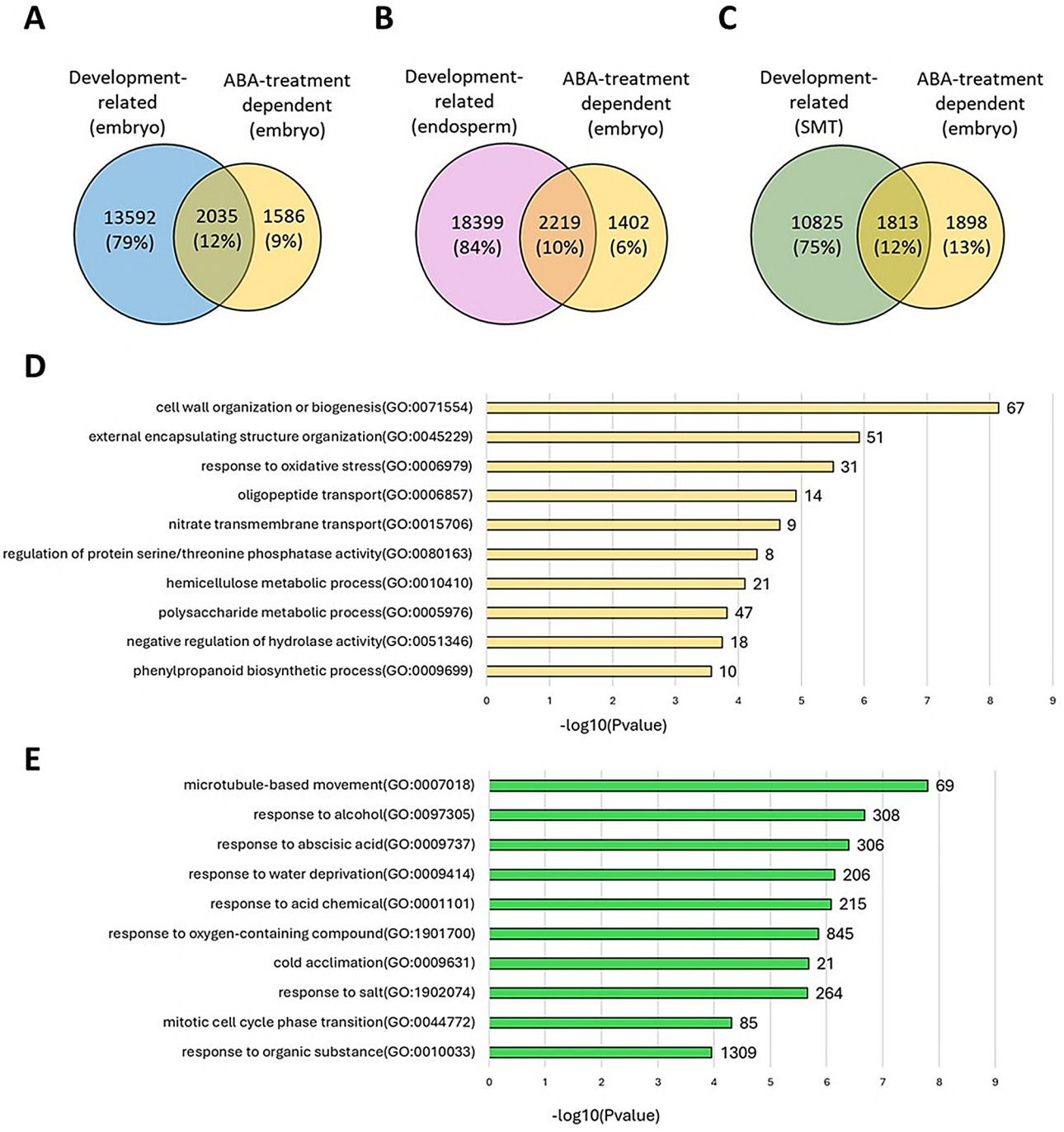


**Fig. 8** Venn diagram illustrating the common and unique differentially expressed genes (DEGs) in barley embryos germinating under 75 µM ABA treatment, in comparison to DEGs in (**A**) embryo, (**B**) endosperm, (**C**) seed maternal tissue (SMT) during seed development. (**D**) Overrepresented GO biological processes of ABA-treatment dependent DEGs in embryo. (**E**) Overrepresented GO biological processes of ABA-treatment dependent and development-related DEGs in embryo

assessed. After ABA treatment, the expression of *GA3* (*ent-kaurene oxidase*), which catalyzes the three consecutive steps of GA biosynthesis, converting ent-kaurene to ent-kaurenic acid, was increased. In addition, the expression of *GA20ox* (*GA20-oxidase*) and *GA2ox* (*GA2-oxidase*) genes was upregulated. GA20ox plays a key role in biosynthesis, converting precursors to active forms of GA, whereas GA2ox is involved in catabolism, inactivating GA [68, 69]. Despite the upregulated expression of genes associated with both pathways, the levels of gibberellins GA12, GA12-aldehyde, GA44, GA15, and GA24 were reduced and negatively correlated with ABA. This

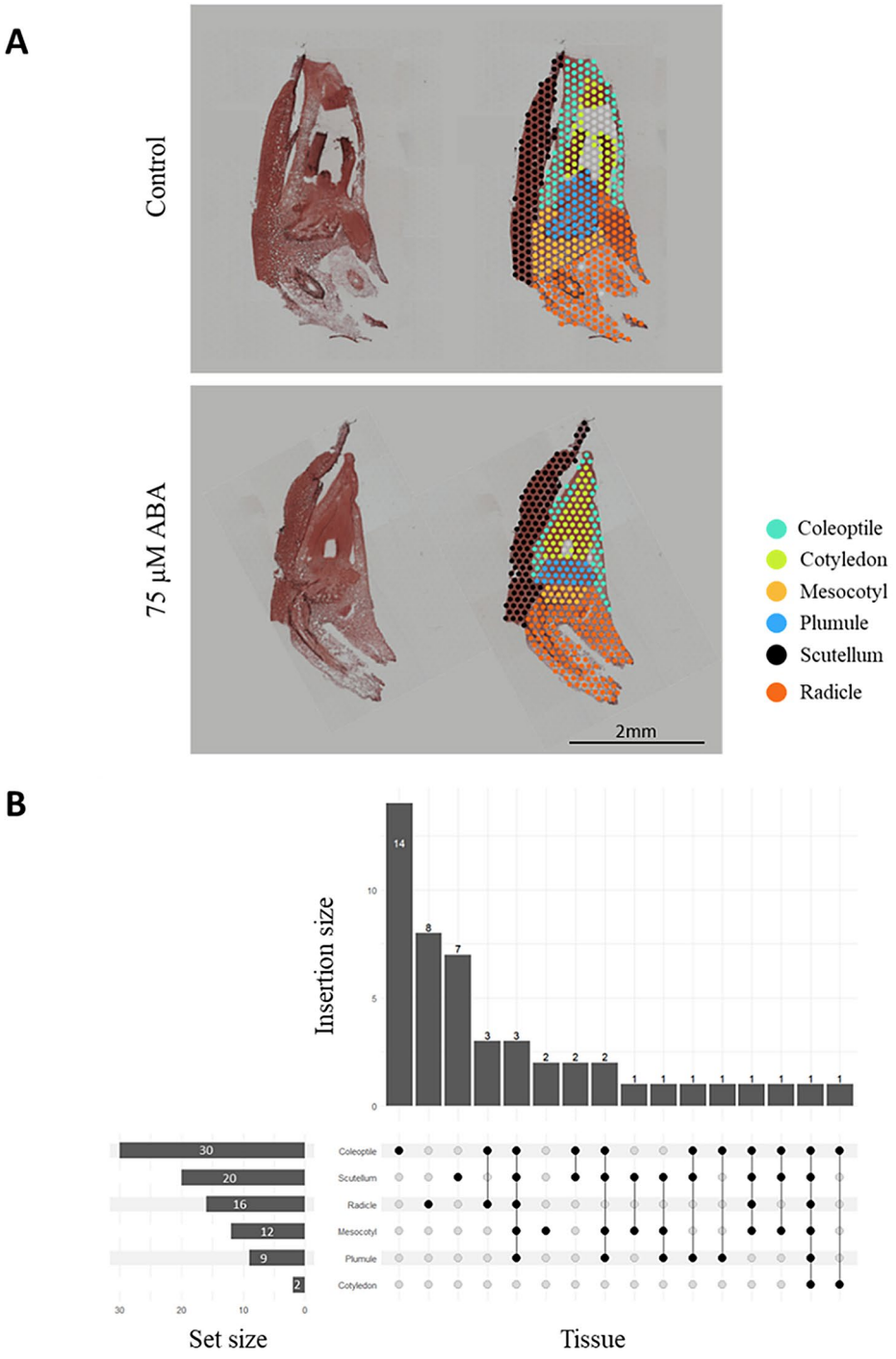


**Fig. 9** Spatial transcriptomics analysis of germinating embryos under control conditions and in the presence of ABA. (**A**) Histological visualization of embryos and spatial representation of cluster localization. (**B**) Distribution of ABA-treatment dependent differentially expressed genes (DEGs) detected in RNA-seq and spatial transcriptomics experiment across embryo tissues

suggests that GA catabolism may predominate over biosynthesis. Interestingly, the expression of the gibberellin receptor, *GID1*, which is responsible for the perception of the active forms of GA, was positively correlated with ABA. It is possible that the upregulation of *GID1* at elevated ABA levels may act as a compensatory mechanism, preparing the seeds for a rapid response to GA signals after the stress conditions have declined and the ABA levels decrease.

In addition to GA, our results revealed a relationship between ABA and other phytohormones. ABA treatment led to decreased levels of JA, which may be due to the

**Table 5** Expression profiles of ABA-dependent DEGs in RNA-seq and Spatial transcriptomics across germinating embryo tissues

| BaRTv2 ID | log2FC | Gene annotation | log2FC | | | | | |
|---|---|---|---|---|---|---|---|---|
| | RNA-seq | | Coleoptile | Cotyledon | Mesocotyl | Plumule | Scutellum | Radicle |
| BaRT2v18chr7HG337790 | 8.59 | GDSL esterase/lipase | 6.95 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr1HG031670 | 4.82 | Wound-induced protease inhibitor | -2.80 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr5HG240300 | 3.30 | Asparagine synthetase | 4.54 | 7.54 | 3.82 | 6.94 | 5.67 | 3.70 |
| BaRT2v18chr3HG162300 | 2.99 | Selenium-binding protein | 1.91 | #N/D | 2.87 | 4.24 | 2.36 | 3.65 |
| BaRT2v18chr3HG124910 | 2.42 | Clavaminate synthase-like protein | 3.66 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr7HG342110 | 2.40 | Sucrose synthase | #N/D | #N/D | -1.48 | #N/D | #N/D | #N/D |
| BaRT2v18chr2HG059360 | 2.33 | Hypoxia-responsive family protein-like | 1.17 | #N/D | 0.83 | 0.77 | 1.27 | #N/D |
| BaRT2v18chr3HG149970 | 2.03 | Aspartate aminotransferase | #N/D | #N/D | #N/D | #N/D | -0.45 | #N/D |
| BaRT2v18chr7HG365400 | 1.97 | Phosphatidylinositol 4-kinase gamma 4 | 0.69 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr1HG047280 | 1.77 | Glucose-1-phosphate adenylyltransferase | 2.97 | #N/D | #N/D | 3.16 | 2.36 | #N/D |
| BaRT2v18chr5HG264980 | 1.75 | Sulfate adenylyltransferase | 3.74 | #N/D | 0.89 | 3.18 | 2.13 | 2.65 |
| BaRT2v18chr3HG145450 | 1.71 | Glycosyltransferase | 8.50 | #N/D | 3.78 | 6.68 | 4.97 | 1.39 |
| BaRT2v18chr3HG141170 | 1.66 | Siroheme synthase | 3.68 | #N/D | 1.96 | 5.43 | 2.28 | #N/D |
| BaRT2v18chr3HG155900 | 1.63 | Early response to dehydration 15-like protein | 1.56 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr5HG237100 | 1.58 | Pyruvate kinase | #N/D | #N/D | #N/D | #N/D | -0.41 | #N/D |
| BaRT2v18chr1HG013760 | 1.55 | Actin cross-linking protein | -0.68 | #N/D | #N/D | #N/D | #N/D | -1.49 |
| BaRT2v18chr7HG376830 | 1.55 | Glycosyltransferase | 5.07 | #N/D | #N/D | #N/D | 3.79 | #N/D |
| BaRT2v18chr4HG209640 | 1.51 | Evolutionarily conserved C-terminal region 2 | 1.28 | #N/D | #N/D | 1.35 | #N/D | #N/D |
| BaRT2v18chr2HG086550 | -1.60 | Aldose 1-epimerase | -2.16 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr4HG179080 | -1.62 | UDP-glucose 6-dehydrogenase 4 | -1.57 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr2HG064340 | -1.67 | Chymotrypsin inhibitor | #N/D | #N/D | #N/D | #N/D | #N/D | 1.42 |
| BaRT2v18chr2HG072330 | -1.77 | Xyloglucan endotransglucosylase/hydrolase | -0.52 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr5HG232080 | -1.82 | Acyl carrier protein | -1.35 | -1.73 | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr7HG373140 -BaRT2v18chr7HG373150 | -2.04 | Peptidyl-prolyl cis-trans isomerase | -0.31 | #N/D | #N/D | #N/D | #N/D | -0.99 |
| BaRT2v18chr2HG064310 | -2.12 | Chymotrypsin inhibitor | #N/D | #N/D | #N/D | #N/D | #N/D | 2.44 |
| BaRT2v18chr4HG195760 | -2.13 | RNA-binding protein | -1.31 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr7HG365210 | -2.14 | Chaperone DnaJ | -1.50 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr1HG031720 | -2.29 | Phospholipase A1 | -1.02 | #N/D | #N/D | #N/D | #N/D | -0.55 |
| BaRT2v18chr2HG058180 | -2.29 | Stress responsive protein | #N/D | #N/D | #N/D | #N/D | -4.71 | #N/D |
| BaRT2v18chr5HG269020 | -2.45 | Agglutinin domain-containing protein | 0.94 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr6HG320150 | -2.47 | Alpha-amylase | #N/D | #N/D | #N/D | #N/D | -0.81 | #N/D |
| BaRT2v18chr3HG163760 | -2.68 | Metacaspase-1 | -2.76 | #N/D | -2.31 | #N/D | -1.27 | -0.92 |
| BaRT2v18chr7HG374840 | -2.72 | Xyloglucan endotransglucosylase | #N/D | #N/D | #N/D | #N/D | -2.89 | #N/D |

**Table 5** (continued)

| BaRTv2 ID | log2FC | Gene annotation | log2FC | | | | | |
|---|---|---|---|---|---|---|---|---|
| | RNA-seq | | Coleoptile | Cotyledon | Mesocotyl | Plumule | Scutellum | Radicle |
| BaRT2v18chr3HG170990 | -2.91 | Abscisic stress ripening protein | #N/D | #N/D | -2.76 | #N/D | -2.37 | #N/D |
| BaRT2v18chr1HG045950 | -2.92 | Subtilisin-like protease | #N/D | #N/D | -3.03 | -3.81 | -2.96 | #N/D |
| BaRT2v18chr3HG123190 | -3.01 | Inhibitor protein | #N/D | #N/D | #N/D | #N/D | #N/D | -0.61 |
| BaRT2v18chr4HG220400 | -3.04 | Metacaspase-1 | -2.41 | #N/D | -3.16 | #N/D | -1.29 | #N/D |
| BaRT2v18chr3HG174080 | -3.17 | Jasmonate induced protein | #N/D | #N/D | #N/D | #N/D | -4.22 | #N/D |
| BaRT2v18chr1HG016260 | -3.85 | Ribulose bisphosphate carboxylase small chain | -2.31 | #N/D | #N/D | #N/D | -3.62 | #N/D |
| BaRT2v18chr1HG041160 | -3.93 | Metal ion binding protein | #N/D | #N/D | #N/D | #N/D | -1.09 | #N/D |
| BaRT2v18chr1HG003900 | -4.07 | Cinnamyl-alcohol dehydrogenase | -2.39 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr2HG062560 | -4.13 | Cold shock protein | #N/D | #N/D | #N/D | #N/D | #N/D | -4.58 |
| BaRT2v18chr1HG007760 | -4.35 | Annexin | #N/D | #N/D | -5.35 | #N/D | #N/D | #N/D |
| BaRT2v18chr7HG371000 | -4.59 | Germin-like protein 8–4 | -1.41 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr7HG378630 | -4.60 | Chitinase | #N/D | #N/D | #N/D | #N/D | #N/D | -4.76 |
| BaRT2v18chr1HG003860 | -4.69 | Glycosyltransferase | -2.06 | #N/D | #N/D | #N/D | #N/D | #N/D |
| BaRT2v18chr6HG281850 | -4.97 | Thionin | #N/D | #N/D | #N/D | #N/D | #N/D | -2.16 |
| BaRT2v18chr7HG374060 | -5.35 | Endoglucanase | #N/D | #N/D | #N/D | #N/D | #N/D | -5.74 |
| BaRT2v18chr1HG001750 | -5.73 | Thionin-2.2 | #N/D | #N/D | #N/D | #N/D | #N/D | -6.27 |

observed decreases in jasmonate precursors such as linolenic acid, 13-HPOTE (13-hydroperoxyoctadecatrienoic acid) and OPDA (12-oxophytodienoic acid). Reduced expression of *LOX* genes encoding lipoxygenases, which convert linolenic acid to 13(S)-hydroperoxyoctadecatrienoic acid (13-HPOTE), suggests a lower activity of these enzymes, resulting in reduced production of 13-HPOTE. This compound is converted into 12-oxo-phytodienoic acid (OPDA), a direct precursor of JA. Thus, due to the decreased level of 13-HPOTE, there was also a decrease in OPD. As a result, even with the increased expression of genes encoding 12-oxophytodienoate reductase (OPR), which is responsible for the further steps of OPDA conversion to JA, the lack of precursor metabolites effectively blocks the entire JA production pathway. This is an intriguing result, as research indicates that JA enhances ABA function and that JA biosynthetic gene expression and JA levels increase in response to ABA [70, 71]. This could also be suggested from the positive correlations between ABA and *COI1*, an F-box protein that promotes the transcriptional repression of JAZ [72]. However, some studies have suggested antagonistic roles of ABA and JA. In wheat grains, MeJA inhibits the expression of an ABA biosynthetic gene (*TaNCED1*), reducing ABA levels and releasing dormancy [24]. In Arabidopsis, JA and its precursor OPDA inhibit seed germination, suggesting different JA functions depending on the species [22]. Further, besides activating JA signaling, COI1 can also inhibit ABA-mediated responses through the interaction and repression of transcriptional activation of ABI3 and ABI5 [25]. This was consistent with the increased expression of *ABI3* (*BaRT2v18chr3HG161790*) in our study. Clearly, the exact role of this interaction needs to be studied further.

In the present study, we observed a negative correlation between ABA and AUX-related genes after ABA treatment at the transcriptomic level. ABA has an inhibitory effect on key auxin genes, limiting both biosynthesis genes (*BaRT2v18chr3HG123080* and *BaRT2v18chr2HG108970*), transport (*BaRT2v18chr4HG185730*), signaling (*BaRT2v18chr7HG339240*), and the early response to this phytohormone (*BaRT2v18chr2HG05972*, *BaRT2v18chr6HG292090*). In contrast, a positive correlation between ABA and two AUX-related genes (*BaRT2v18chr2HG105230* and *BaRT2v18chr3HG147750*) was observed. Furthermore, reduced expression of genes from the tryptophan pathway, an essential precursor of auxin, suggests that ABA inhibits the expression of genes related to AUX production. However, our data do not indicate a statistically significant increase or decrease in the level of AUX metabolites after ABA treatment. This may suggest that the observed changes in gene expression reflect local, tissue-specific changes in AUX signaling or metabolism, rather than global changes in their endogenous levels. Interestingly, recent studies have shown that exogenous AUX can act

synergistically with JA, enhancing the effect of ABA and delaying germination by modulating the transcription factors AUXIN RESPONSE FACTOR 10 (ARF10) and ARF16 [26, 27]. However, the effect of AUX on germination is dose-dependent and it can both stimulate and inhibit this process [73–76]. What is more, Belin et al. (2009) showed that low concentrations of ABA (2 μM) induce the expression of the *ProIAA2:GUS* marker, while higher concentrations (30 μM) strongly inhibit it [77]. ABA also limits the expression of genes encoding proteins responsible for auxin transport, both the influx carrier *AUXIN RESISTANT 1* (*AUX1*) and the efflux carriers *PIN-FORMED 3* (*PIN3)* and *PIN7*, even under light conditions that usually stimulate their activity [78]. Although the role of AUX in germination is still not fully understood, the obtained results highlight the complexity of the interaction between ABA and AUX.

Campesterol, the precursor of BR, plays a key role in flux through the subsequent steps of the BR pathway [79]. The reduction of its level in our metabolomics data, as well as of active BR such as brassinolide and castasterone, suggests that ABA has an inhibitory effect on BR biosynthesis at its early stages. BRs are known to promote seed germination; therefore, their deficiency supports the action of ABA as an inhibitor of this process and maintains seed dormancy [80–83]. In addition, the reduced expression of genes annotated as *CYP92A6*, a key BR biosynthesis gene, and *BAS1* (*CYP734A1*), involved in BR inactivation, suggests that ABA regulates BR homeostasis by modulating both their synthesis and inactivation pathways [84, 85].

Our observations indicate that ABA inhibits the conversion of 1-aminocyclopropane-1-carboxylate (ACC) to ET by reducing the activity of ACC oxidase (ACO) and decreasing the accumulation of its transcripts, which is consistent with previous studies [86]. Our data showing reduced expression of genes related to the synthesis and metabolism of ACC and its precursors, such as *S-adenosylmethionine synthase* (*SAM synthase*; *BaRT2v18chr6HG310120*) and *methionine adenosyltransferase* (*BaRT2v18chr6HG310160*), as well as genes encoding key enzymes of ET biosynthesis, *ACC synthase* (*ACS*; *BaRT2v18chr3HG124710*, *BaRT2v18chr2HG095020*), and *ACC oxidase* (*ACO*; *BaRT2v18chr5HG250670*, *BaRT2v18chr4HG184710*, *BaRT2v18chr6HG319390*). We also observed a positive association between ABA treatment and the expression of homologs of the ET-activated transcription factor *EIN3* (*BaRT2v18chr2HG086440*) and the ET receptor *ERS2* (*BaRT2v18chr6HG314730*). This suggests a two-sided effect of ABA; on the one hand, ABA inhibits the expression of genes involved in ET synthesis, limiting its production, whereas the plant signaling apparatus may be prepared to respond to this phytohormone. Although the effect of ABA on ET signaling is poorly understood, other studies have indicated that ET regulates seed germination by reducing ABA levels and attenuating ABA signaling. Mutations that reduce ET sensitivity (*etr1*, *ein2*, *ein6*) increase ABA sensitivity and inhibit germination, whereas mutations that increase ET sensitivity (*ctr1*, *eto1*) reduce ABA action, promoting germination [87–89]. However, mutations in ET signaling pathway genes, such as *EIN3*, *EIN4*, *EIN5*, and *EIN7* do not significantly affect ABA sensitivity [89]. Additionally, genes encoding the ET receptors *ERS1* and *ERS2* do not play a significant role in modulating ABA signaling or in ET-related responses in the context of seed germination [90].

Studies have shown that ABA treatment leads to a significant reduction in the expression of CTK signaling genes *ARABIDOPSIS RESPONSE REGULATOR 6* (*ARR6*), *ARR7* and *ARR15* during seed germination [91]. ABA regulates transcription by activating the transcription factor ABI4, which directly binds to its promoters and inhibits their expression. Therefore, the downregulation of A-ARR genes observed in our study is consistent with the mechanism by which ABA suppresses CTK signaling and promotes the inhibition of seed germination. Moreover, ABA has been shown that ABA can affect CTK biosynthesis by decreasing the expression of biosynthetic genes such as *ISOPENTENYLTRANSFERASE 3* (*IPT3*) and *IPT8* [21]. The reduced expression of genes involved in CTK degradation (*BaRT2v18chr1HG019230*; *AtCKX5*), biosynthetic enzymes (*BaRT2v18chr5HG246980*; *AtCYP735A1*), and glucosylating enzymes (*BaRT2v18chr2HG096460*; *AtUGT72E1*, *BaRT2v18chr2HG096430*; *AtUGT84A3*) observed in our study suggest a modulation of the balance between active and inactive forms of CTK at the transcriptomic level in response to ABA.

Our study also suggests that strigolactones play an important role in controlling ABA-dependent seed germination. We observed that the level of sorgolactone, a specific type of strigolactone, was negatively correlated with ABA, and its concentration significantly decreased in germinating embryos after ABA treatment [92]. It is also worth noting that ABA and salicylic acid (SA) are phytohormones with opposite functions [93]. Similarly, the observed negative correlation between SA levels and *PR1* gene expression (*BaRT2v18chr5HG244050*) supported the hypothesis of an antagonistic interaction between these two phytohormones.

Comparisons of expression patterns of three developing seed tissues (embryo, endosperm, and SMT) described by Kovacik et al. (2024) showed that only a small number (from 10 to 12%) of DEGs overlap with those identified in our study [44]. This limited overlap suggests that ABA can induce a transcriptional response shaped by a common developmental ABA regulatory core, while also

including tissue-specific regulation. Analysis of the biological processes specific to germinating embryos treated with ABA revealed that exogenous ABA application regulates adaptive and structural mechanisms in barley embryos, such as cell wall modification, hemicellulose and polysaccharide metabolism, and responses to oxidative stress and phenylpropanoid biosynthesis. This suggests that the role of exogenous ABA is not limited solely to triggering adaptation in response to abiotic stress but also includes key functions in the regulation of normal developmental processes [94–96].

To further refine our understanding of these spatially distinct regulatory processes, we applied Visium spatial transcriptomics (10× Genomics). Unlike bulk transcriptome analysis, which captures averaged gene expression across mixed cell populations, this approach enabled precise mapping of the gene expression in specific embryo tissues, uncovering spatial patterns critical for understanding localized responses to ABA. We localized the expression of 49 genes, selected from the pool of 1,586 ABA-treatment-dependent DEGs identified in bulk RNA-seq, across six embryonic tissues, such as coleoptile, cotyledon, mesocotyl, plumule, scutellum, and radicle. Our results highlighted the important role of the coleoptile tissue, which exhibited the greatest overlap of DEGs with other embryo tissues and expressed the highest number of tissue-specific genes (14 DEGs). This suggests its dual function as both a hub for shared stress-responsive genes and a site of unique gene expression patterns. The obtained results show that ABA responses are closely related to tissue localization, which may reflect the different physiological roles of individual tissues for embryo development. It is noteworthy that spatial transcriptomics revealed differential gene expression distributions that would otherwise remain masked in bulk RNA-seq data, offering a refined view of tissue-specific ABA responses and their functional implications during germination. This level of resolution underscores the importance of spatial context in interpreting the functional roles of ABA-responsive genes.

## Conclusions

Using bulk transcriptomics, metabolomics and Visium spatial transcriptomics, we provide first spatially resolved, multi-omic map of barley seed germination under exogenous ABA. The data indicate that ABA limits germination by coordinating its own signalling–metabolite module and by interacting with GA, JA, BR, SA and auxin pathways. Spatial mapping additionally points to the coleoptile as a principal site of ABA-responsive transcription, a pattern not visible in bulk datasets. The resulting list of tissue-specific genes and metabolites associated with growth restraint and stress adaptation provides a useful reference for future physiological and breeding studies.

**Abbreviations**

| | |
|---|---|
| ABA | Abscisic acid |
| AUX | Auxin |
| BR | Brassinosteroid |
| CTK | Cytokinin |
| DAI | Day after imbibition |
| DEGs | Differentially expressed genes |
| ET | Ethylene |
| GA | Gibberellic acid |
| GO | Gene ontology |
| JA | Jasmonic acid |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| SA | Salicylic acid |
| TF | Transcription factor |
| TPM | Transcript per million |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06654-z.

Supplementary Material 1: **Additional file 1: Data S1.** List of differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions. (XLSX 1 302 KB)

Supplementary Material 2: **Additional file 2: Data S2.** List of the differentially expressed transcription factors in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions. (XLSX 28 KB)

Supplementary Material 3: **Additional file 3: Data S3.** Differentially expressed transcription factors, with binding sites among the differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions. (XLSX 3 982 KB)

Supplementary Material 4: **Additional file 4: Data S4.** GO biological process analysis of differentially upregulated /downregulated genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions. (XLSX 77 KB)

Supplementary Material 5: **Additional file 5: Data S5.** List of genes in top overrepresented GO biological process of differentially upregulated / downregulated genes in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions. (XLSX 80 KB)

Supplementary Material 6: **Additional file 6: Data S6.** Differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions within the response to abscisic acid (GO:0009737) Gene Ontology Biological Process (GO-BP). (XLSX 14 KB)

Supplementary Material 7: **Additional file 7: Data S7.** Functional enrichment of differentially expressed transcription factors in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions based on the KEGG pathway category. (XLSX 14 KB)

Supplementary Material 8: **Additional file 8: Data S8.** List of upregulated and downregulated differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions within the Plant Hormone Signal Transduction map (KEGG). (XLSX 15 KB)

Supplementary Material 9: **Additional file 9: Data S9.** List of upregulated and downregulated differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions within the biosynthesis of plant hormones pathway maps. (XLSX 14 KB)

Supplementary Material 10: **Additional file 10: Data S10.** Identified phytohormones and metabolites in germinating barley embryos at 1 DAI under 75 µM ABA treatment and control conditions. (XLSX 17 KB)

Supplementary Material 11: **Additional file 11: Data S11.** P-value correlations between metabolomic and transcriptomic data of germinating barley embryos at 1 DAI after 75 µM ABA treatment and in control conditions. (XLSX 37 KB)

Supplementary Material 12: **Additional file 12: Data S12.** List of ABA-treatment dependent differentially expressed genes (DEG) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions. (XLSX 410 KB)

Supplementary Material 13: **Additional file 13: Data S13.** GO biological process analysis of ABA-treatment dependent differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions. (XLSX 24 KB)

Supplementary Material 14: **Additional file 14: Data S14.** Differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions in ABA-treatment dependent overrepresented GO biological processes. (XLSX 29 KB)

Supplementary Material 15: **Additional file 15: Data S15.** List of ABA-treatment dependent and development-related differentially expressed genes (DEG) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions and in embryos during seed development. (XLSX 651 KB)

Supplementary Material 16: **Additional file 16: Data S16.** GO biological process analysis of ABA-treatment dependent and development-related differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions. (XLSX 38 KB)

Supplementary Material 17: **Additional file 17: Data S17.** Differentially expressed genes (DEGs) in germinating barley embryos at 1 DAI after 75 µM ABA treatment compared to control conditions in ABA-treatment dependent and development-related overrepresented GO biological processes. (XLSX 43 KB)

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**Author contributions**
ADG Conceptualization; ADG, ES Experimental design; ADG, ES, SH, JSZ, MK, AP research analysis; BH, LM, MB metabolome analysis; ES investigation and data interpretation, ADG data curation; ES, ADG writing–original draft; ADG, ES, BSH, LM, SH, JSZ, AP writing–review and editing; ES, BH visualization; ADG supervision; ADG project administration; and ADG Funding acquisition.

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**Data availability**
Data generated or analyzed during this study are included in this published article (and its additional files). The RNA-seq data used in the present study were deposited into EMBL-EBI (EMBL's European Bioinformatics Institute) in the Array Express repository (https://www.ebi.ac.uk/) under the accession number E-MTAB-13989. The spatial transcriptomic data used in the present study have been deposited into EMBL-EBI (EMBL's European Bioinformatics Institute) in the Array Express repository (https://www.ebi.ac.uk/) under accession number E-MTAB-14835. Transcriptome data from developing seed tissues, used in the comparative analysis, were obtained from the supplementary materials of the study by Kovacik et al. (2024) [44]. The RNA-seq data generated in the study by Kovacik et al. (2024) are available in the Gene Expression Omnibus (GEO) at https://www.ncbi.nlm.nih.gov/geo/ under accession number GSE233316.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

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

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miejscowość, data

Ewa Sybilska
imię i nazwisko kandydata

Instytut Biologii, Biotechnologii i Ochrony Środowiska,
Wydział Nauk Przyrodniczych,
Uniwersytet Śląski w Katowicach
afilacja

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która jest integralną częścią cyklu prac, składających się na moją rozprawę doktorską, polegał na zebraniu i analizie danych literaturowych, napisaniu oryginalnej wersji manuskryptu i przygotowaniu wersji końcowej, wykonaniu tabel i figur, korekcie wersji oryginalnej i końcowej manuskryptu oraz przygotowaniu odpowiedzi na uwagi recenzentów.

Ewa Sybilska
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miejscowość, data

Agata Daszkowska-Golec
imię i nazwisko współautora publikacji

Instytut Biologii, Biotechnologii i Ochrony Środowiska,
Wydział Nauk Przyrodniczych,
Uniwersytet Śląski w Katowicach
afilacja

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która jest integralną częścią cyklu prac, składających się na rozprawę doktorską Ewy Sybilskiej, polegał na wsparciu w opracowaniu koncepcji i struktury manuskryptu oraz udziale w przygotowaniu manuskryptu na każdym etapie. Ponadto manuskrypt powstał w ramach projektu badawczego, na który uzyskałam finansowanie i którym kieruję. Jestem autorem korespondencyjnym.

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z dnia 19 lutego 2024 r.

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miejscowość, data

Ewa Sybilska
imię i nazwisko kandydata

Instytut Biologii, Biotechnologii i Ochrony Środowiska,
Wydział Nauk Przyrodniczych,
Uniwersytet Śląski w Katowicach
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która jest integralną częścią cyklu prac, składających się na moją rozprawę doktorską, polegał na udziale w zaprojektowaniu eksperymentu, przeprowadzeniu wszystkich analiz laboratoryjnych, bioinformatycznej analizie danych, integracji danych, przeprowadzeniu analizy statystycznej, interpretacji wyników, pisaniu oryginalnej wersji manuskryptu i przygotowaniu wersji końcowej, wykonaniu tabel i figur, oraz formułowaniu odpowiedzi na uwagi recenzentów.

Ewa Sybilska
.....

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Prorektora ds. nauki i finansów
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miejscowość, data

Anna Collin
imię i nazwisko współautora publikacji

Instytut Biologii, Biotechnologii i Ochrony Środowiska,
Wydział Nauk Przyrodniczych,
Uniwersytet Śląski w Katowicach
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Oświadczam, że mój wkład do pracy:

Sybilska, E., Collin, A., Sadat Haddadi, B., Mur, L. A. J., Beckmann, M., Guo, W., Simpson, C. G., & Daszkowska-Golec, A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (Hordeum vulgare). Scientific Reports, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9

która jest integralną częścią cyklu prac, składających się na rozprawę doktorską Ewy Sybilskiej, polegał na wsparciu w trakcie prowadzenia badań laboratoryjnych oraz weryfikacji treści manuskryptu.

A. Collin

podpis współautora publikacji

123:7488164853

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Aberystwyth, 22.05.2025
location, date

Bahareh Sadat Haddadi
first and last name of co-author of the publication

Department of Life Science,
Aberystwyth University,
Aberystwyth, UK
affiliation

**A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK**

I declare that my contribution to the work:

Sybilska, E., Collin, A., Sadat Haddadi, B., Mur, L. A. J., Beckmann, M., Guo, W., Simpson, C. G., & Daszkowska-Golec, A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (Hordeum vulgare). Scientific Reports, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9

which is an integral part of the series of publications constituting Ewa Sybilska's doctoral dissertation, consisted of performing metabolome analysis, and reviewing all versions of the manuscript.

Bahar Haddadi 22.05.2025

signature of the co-author of the publication

124:4314493989

PRIFYSGOL ABERYSTWYTH UNIVERSITY

IBERS
Sefydliad y Gwyddorau Biolegol, Amgylcheddol a Gwledig
Institute of Biological, Environmental and Rural Sciences

Adeilad Edward Llwyd,
Campws Penglais
Aberystwyth
Ceredigion SY23 3DA
Athro Luis A. J. Mur
Ffôn: (01970) 622981
Ffacs: (01970) 622350
Ebost: lum@aber.ac.uk
www.aber.ac.uk/ibers

Edward Llwyd Building
Penglais Campus
Aberystwyth
Ceredigion SY23 3DA
Prof. Luis A. J. Mur
Tel: (01970) 622981
Fax: (01970) 622350
Email: lum@aber.ac.uk
www.aber.ac.uk/ibers

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.
Aberystwyth, 22.05.2025

Luis A. J. Mur
Department of Life Science,
Aberystwyth University,
Aberystwyth, UK

**A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK**

I declare that my contribution to the work:

Sybilska, E., Collin, A., Sadat Haddadi, B., Mur, L. A. J., Beckmann, M., Guo, W., Simpson, C. G., & Daszkowska-Golec, A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (Hordeum vulgare). Scientific Reports, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9

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PRIFYSGOL ABERYSTWYTH UNIVERSITY

.....

signature of the co-author of the publication

THE QUEEN'S ANNIVERSARY PRIZES 2009

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*Yn unol â pholisi dwyieithog Prifysgol Aberystwyth, mae croeso i chi ohebu â'r Brifysgol yn Gymraeg neu yn Saesneg.*
In accordance with the bilingual policy of Aberystwyth University, you are welcome to correspond with the University in Welsh or in English.

125:1516399970

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Aberystwyth, 22.05.2025
location, date

Manfred Beckmann
first and last name of co-author of the publication

Department of Life Science,
Aberystwyth University,
Aberystwyth, UK
affiliation

**A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK**

I declare that my contribution to the work:

Sybilska, E., Collin, A., Sadat Haddadi, B., Mur, L. A. J., Beckmann, M., Guo, W., Simpson, C. G., & Daszkowska-Golec, A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (Hordeum vulgare). Scientific Reports, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9

which is an integral part of the series of publications constituting Ewa Sybilska's doctoral dissertation, consisted of performing liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for subsequent metabolome analysis.

……
signature of the co-author of the publication

126:6935713704

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Dundee, 22.05.2025
location, date

Wenbin Guo
first and last name of co-author of the publication

Information and Computational Sciences,
James Hutton Institute,
Dundee, DD2 5DA, Scotland, UK
affiliation

**A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK**

I declare that my contribution to the work:

Sybilska, E., Collin, A., Sadat Haddadi, B., Mur, L. A. J., Beckmann, M., Guo, W., Simpson, C. G., & Daszkowska-Golec, A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (Hordeum vulgare). Scientific Reports, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9

which is an integral part of the series of publications constituting Ewa Sybilska's doctoral dissertation, consisted of the integration of RNA-seq and Iso-Seq data, construction of the BarkeRTD reference transcriptome dataset, preparation of Figure 6, and reviewing the manuscript content.

Wenbin Guo
.....
signature of the co-author of the publication

127:9817366027

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Dundee, 22.05.2025

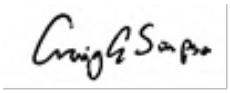
Craig G. Simpson
Honorary Associate,
James Hutton Institute,
Dundee, DD2 5DA, Scotland, UK

**A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK**

I declare that my contribution to the work:

Sybilska, E., Collin, A., Sadat Haddadi, B., Mur, L. A. J., Beckmann, M., Guo, W., Simpson, C. G., & Daszkowska-Golec, A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (Hordeum vulgare). Scientific Reports, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9

which is an integral part of the series of publications constituting Ewa Sybilska's doctoral dissertation, consisted of editing and verification of the manuscript content.



……
signature of the co-author of the publication

128:3941088349

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Katowice, 22.05.2025
miejscowość, data

Agata Daszkowska-Golec
imię i nazwisko współautora publikacji

Instytut Biologii, Biotechnologii i Ochrony Środowiska,
Wydział Nauk Przyrodniczych,
Uniwersytet Śląski w Katowicach
afilacja

**OŚWIADCZENIE WSPÓŁAUTORA OSOBY UBIEGAJĄCEJ SIĘ O WŁASNYM WKŁADZIE W POWSTAWANIE PRACY**

Oświadczam, że mój wkład do pracy:

Sybilska, E., Collin, A., Sadat Haddadi, B., Mur, L. A. J., Beckmann, M., Guo, W., Simpson, C. G., & Daszkowska-Golec, A. (2024). The cap-binding complex modulates ABA-responsive transcript splicing during germination in barley (Hordeum vulgare). Scientific Reports, 14(1), 18278. https://doi.org/10.1038/s41598-024-69373-9

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.....

podpis współautora publikacji

129:4093986823

Załącznik nr 9
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Katowice, 22.05.2025
miejscowość, data

Ewa Sybilska
imię i nazwisko kandydata

Instytut Biologii, Biotechnologii i Ochrony Środowiska,
Wydział Nauk Przyrodniczych,
Uniwersytet Śląski w Katowicach
afilacja

**OŚWIADCZENIE OSOBY UBIEGAJĄCEJ SIĘ O WŁASNYM WKŁADZIE W POWSTAWANIE PRACY**

Oświadczam, że mój udział w pracy:

Sybilska, E., Haddadi, B. S., Mur, L. A. J., Beckmann, M., Hryhorowicz, S., Suszynska-Zajczyk, J., Knaur, M., Pławski, A., & Daszkowska-Golec, A. (2025). Mapping the molecular signature of ABA-regulated gene expression in germinating barley embryos. BMC Plant Biology, 25(1), 619. https://doi.org/10.1186/s12870-025-06654-z

która jest integralną częścią cyklu prac, składających się na moją rozprawę doktorską, polegał na udziale w zaprojektowaniu eksperymentu, przeprowadzeniu wszystkich badań laboratoryjnych, udziale w optymalizacji technologii Visium Spatial Gene Expression (10× Genomics) oraz dostosowaniu protokołu do specyfiki badanego materiału, bioinformatycznej analizie danych, interpretacji wyników, pisaniu oryginalnej wersji manuskryptu i przygotowaniu wersji końcowej, wykonaniu tabel i figur, oraz formułowaniu odpowiedzi na uwagi recenzentów.

Ewa Sybilska
.....

podpis

130:1033326670

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Aberystwyth, 22.05.2025
location, date

Bahareh Sadat Haddadi
first and last name of co-author of the publication

Department of Life Science,
Aberystwyth University,
Aberystwyth, UK
affiliation

**A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK**

I declare that my contribution to the work:

Sybilska, E., Haddadi, B. S., Mur, L. A. J., Beckmann, M., Hryhorowicz, S., Suszynska-Zajczyk, J., Knaur, M., Pławski, A., & Daszkowska-Golec, A. (2025). Mapping the molecular signature of ABA-regulated gene expression in germinating barley embryos. BMC Plant Biology, 25(1), 619. https://doi.org/10.1186/s12870-025-06654-z

which is an integral part of the series of publications constituting Ewa Sybilska's doctoral dissertation, consisted of metabolome analysis, metabolome results visualization, and reviewing all versions of the manuscript.

Bahar Haddadi 22.5.25

signature of the co-author of the publication

131:6979777163

PRIFYSGOL ABERYSTWYTH UNIVERSITY

IBERS
Sefydliad y Gwyddorau Biolegol, Amgylcheddol a Gwledig
Institute of Biological, Environmental and Rural Sciences

Adeilad Edward Llwyd,
Campws Penglais
Aberystwyth
Ceredigion SY23 3DA
Athro Luis A. J. Mur
Ffôn: (01970) 622981
Ffacs: (01970) 622350
Ebost: lum@aber.ac.uk
www.aber.ac.uk/ibers

Edward Llwyd Building
Penglais Campus
Aberystwyth
Ceredigion SY23 3DA
Prof. Luis A. J. Mur
Tel: (01970) 622981
Fax: (01970) 622350
Email: lum@aber.ac.uk
www.aber.ac.uk/ibers

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.
Aberystwyth, 22.05.2025

Luis A. J. Mur
Department of Life Science,
Aberystwyth University,
Aberystwyth, UK

**A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK**

I declare that my contribution to the work:

Sybilska, E., Haddadi, B. S., Mur, L. A. J., Beckmann, M., Hryhorowicz, S., Suszynska-Zajczyk, J., Knaur, M., Pławski, A., & Daszkowska-Golec, A. (2025). Mapping the molecular signature of ABA-regulated gene expression in germinating barley embryos. BMC Plant Biology, 25(1), 619. https://doi.org/10.1186/s12870-025-06654-z

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PRIFYSGOL ABERYSTWYTH UNIVERSITY

.....

signature of the co-author of the publication

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132:5499528381

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Aberystwyth, 22.05.2025
location, date

Manfred Beckmann
first and last name of co-author of the publication

Department of Life Science,
Aberystwyth University,
Aberystwyth, UK
affiliation

**A STATEMENT OF THE APPLICANT'S CO-AUTHOR OF THEIR CONTRIBUTION TO THE WORK**

I declare that my contribution to the work:

Sybilska, E., Haddadi, B. S., Mur, L. A. J., Beckmann, M., Hryhorowicz, S., Suszynska-Zajczyk, J., Knaur, M., Pławski, A., & Daszkowska-Golec, A. (2025). Mapping the molecular signature of ABA-regulated gene expression in germinating barley embryos. BMC Plant Biology, 25(1), 619. https://doi.org/10.1186/s12870-025-06654-z

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signature of the co-author of the publication

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Poznań, 22.05.2025
miejscowość, data

Szymon Hryhorowicz
imię i nazwisko współautora publikacji

Instytut Genetyki Człowieka,
Polska Akademia Nauk w Poznaniu
afilacja

**OŚWIADCZENIE WSPÓŁAUTORA OSOBY UBIEGAJĄCEJ SIĘ O WŁASNYM WKŁADZIE W POWSTAWANIE PRACY**

Oświadczam, że mój wkład do pracy:

Sybilska, E., Haddadi, B. S., Mur, L. A. J., Beckmann, M., Hryhorowicz, S., Suszynska-Zajczyk, J., Knaur, M., Pławski, A., & Daszkowska-Golec, A. (2025). Mapping the molecular signature of ABA-regulated gene expression in germinating barley embryos. BMC Plant Biology, 25(1), 619. https://doi.org/10.1186/s12870-025-06654-z

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.....

podpis współautora publikacji

134:3866504706

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Poznań, 22.05.2025
miejscowość, data

Joanna Suszyńska-Zajczyk
imię i nazwisko współautora publikacji

[1] Katedra Biochemii i Biotechnologii,
Wydział Rolnictwa, Ogrodnictwa i Biotechnologii,
Uniwersytet Przyrodniczy w Poznaniu
[2] Instytut Genetyki Człowieka,
Polska Akademia Nauk w Poznaniu
afilacja

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Signed by / Podpisano przez: Joanna Suszyńska-Zajczyk
Date / Data: 2025-05-22 08:00

.....

podpis współautora publikacji

135:8194440440

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Poznań, 22.05.2025
miejscowość, data

Monika Knaur
imię i nazwisko współautora publikacji

Instytut Genetyki Człowieka,
Polska Akademia Nauk w Poznaniu
afilacja

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która jest integralną częścią cyklu prac, składających się na rozprawę doktorską Ewy Sybilskiej, polegał na bioinformatycznym przetworzeniu surowych danych, ich przygotowaniu i integracji z narzędziem Loupe Browser w celu dalszych analiz, a także na weryfikacji treści manuskryptu.

Monika Knaur
.....

podpis współautora publikacji

136:3936322387

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Poznań, 22.05.2025
miejscowość, data

Andrzej Pławski
imię i nazwisko współautora publikacji

[1] Instytut Genetyki Człowieka,
Polska Akademia Nauk w Poznaniu
[2] Klinika Chirurgii Ogólnej, Endokrynologicznej i Onkologii Gastroenterologicznej,
Uniwersytet Medyczny im. Karola Marcinkowskiego w Poznaniu
afilacja

**OŚWIADCZENIE WSPÓŁAUTORA OSOBY UBIEGAJĄCEJ SIĘ O WŁASNYM WKŁADZIE W POWSTAWANIE PRACY**

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która jest integralną częścią cyklu prac, składających się na rozprawę doktorską Ewy Sybilskiej, polegał na realizacji współpracy naukowej, dzięki której Kandydatka mogła osobiście uczestniczyć w eksperymencie z zakresu transkryptomiki przestrzennej z wykorzystaniem technologii Visium Spatial Gene Expression (10× Genomics), na sprawowaniu nadzoru nad przebiegiem tego eksperymentu, a także na weryfikacji treści manuskryptu.

Andrzej Pławski
.....

podpis współautora publikacji

137:6517620276

Załącznik nr 10
do pisma okólnego nr 2
Prorektora ds. nauki i finansów
z dnia 19 lutego 2024 r.

Katowice, 22.05.2025
miejscowość, data

Agata Daszkowska-Golec
imię i nazwisko współautora publikacji

Instytut Biologii, Biotechnologii i Ochrony Środowiska,
Wydział Nauk Przyrodniczych,
Uniwersytet Śląski w Katowicach
afilacja

**OŚWIADCZENIE WSPÓŁAUTORA OSOBY UBIEGAJĄCEJ SIĘ O WŁASNYM WKŁADZIE W POWSTAWANIE PRACY**

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.....

podpis współautora publikacji

138:3149128073

## 14. Wykaz aktywności i osiągnięć naukowych

### 14.1. Pozostałe publikacje

1. Sybilska E., Daszkowska-Golec A. (2023). A complex signaling trio in seed germination: Auxin-JA-ABA. *Trends in Plant Science*, 28(8), 873–875. https://doi.org/10.1016/j.tplants.2023.05.003

   $IF_{2023}$: 17,3, MNiSW: 200 pkt

2. Kurowska M., Janiak A., Sitko K., Potocka I., Gajecka M., Sybilska E., Płociniczak T., Lip S., Rynkiewicz M., Wiecha K., Nawrot M., Daszkowska-Golec A., Szarejko I. (2025). Functional analysis of HvSNAC1 in stomatal dynamics and drought adaptation. *Journal of Applied Genetics*. https://doi.org/10.1007/s13353-025-00956-6

   $IF_{2023}$: 2,0, MNiSW: 140 pkt

3. Collin A., Matkowski H., Sybilska E., Biantari A., Król O., Daszkowska-Golec A. (2025). ABA-induced alternative splicing drives transcriptomic reprogramming for drought tolerance in barley. *BMC Plant Biology*, 25(1), 445. https://doi.org/10.1186/s12870-025-06485-y

   $IF_{2023}$: 4,3, MNiSW: 140 pkt

***Łączna suma IF = 23,6***

***Łączna suma punktów MNiSW = 480***

### 14.2 Publikacje w recenzji

1. Sybilska E., Hryhorowicz S., Suszyńska-Zajczyk J., Knaur M., Pławski A., Daszkowska-Golec A. (2025). Spatial transcriptomics uncovered potential role of cap-binding complex in modulation of the ABA-dependent translation and RNA metabolism in germinating barley. Journal of Experimental Botany, manuskrypt w trakcie procesu recenzyjnego

   $IF_{2023}$: 5,8, MNiSW: 140 pkt

***Łączna suma IF = 5,8***

***Łączna suma punktów MNiSW = 140***



139:2430030220

**14.3 Konferencje naukowe**

1. **Sybilska E.**, Haddadi B. S., Mur L. A. J., Beckmann M., Hryhorowicz S., Suszyńska-Zajczyk J., Knaur M., Pławski A., Daszkowska-Golec A. Unraveling ABA's Molecular Signature: Insights into Its Function in Barley Seed Germination. 08-11.07.2025. SEB Centenary Conference 2025, Antwerpia, Belgia, wystąpienie ustne
2. **Sybilska E.**, Collin A., Haddadi B. S., Mur L. A. J., Beckmann M., Guo W., Simpson C. G., Daszkowska-Golec A. The role of Cap-Binding Complex (CBC) in ABA-dependent seed germination in barley. 02-05.07.2024, SEB Centenary Conference 2024, Prague, Czech Republic, poster naukowy
3. **Sybilska E.**, Daszkowska-Golec A. Deciphering the Role of Cap-Binding Complex in ABA-Mediated Seed Germination in Barley. 12.01-17.01.2024, XXXI Plant and Animal Genome Conference, San Diego, California, USA, współautor prezentacji
4. **Sybilska E.**, Daszkowska-Golec A. Molekularne podstawy odpowiedzi podwójnego mutanta *hvcbp20.ab/hvcbp80.b* na ABA podczas kiełkowania nasion u jęczmienia jarego (*Hordeum vulgare*). 09.11-10.11.2023, Dni Młodego Naukowca, Instytut Hodowli i Aklimatyzacji Roślin – PIB, Radzików, Polska, poster naukowy
5. **Sybilska E.**, Daszkowska-Golec A. Transcriptome analysis of CBC mutants reveals the involvement of alternative splicing during seed germination in the presence of ABA in barley. 19.09-22.09.2023, 11th PSEPB Conference, Poznań, Polska, poster naukowy
6. **Sybilska E.**, Fus W., Marzec M., Daszkowska-Golec A. The significance of the Cap-Binding Complex in barley's adaptation to abiotic stress. 04.07-07.07.2023, SEB Centenary Conference 2023, Edynburg, Wielka Brytania, wystąpienie ustne
7. **Sybilska E.**, Fus W., Marzec M., Daszkowska-Golec A. The analysis of barley *cbp20/cbp80* double mutant exposed to drought stress at the seedling stage. 03.07-07.07.2022, 13th International Barley Genetics Symposium, Institute of Agricultural Resources and Economics (AREI), Ryga, Łotwa, poster naukowy& komunikat ustny



140:1178300564

8. Collin A., Matkowski H., **Sybilska E.**, Krol O., Biantari A., Daszkowska-Golec A. The role of ABA in barley response to drought at the pre-flowering stage. 03.07-07.07.2022, 13th International Barley Genetics Symposium, Ryga, Łotwa, współautor posteru naukowego
9. **Sybilska E.**, Marzec M., Daszkowska-Golec A. The response to ABA of barley *hvcbp20.ab/hvcbp80.b* double mutant during seed germination. 29.06-30.06.2022, Reaching Blue skies for a sustainable future, University w Leeds, Wielka Brytania, on-line, poster naukowy& komunikat ustny
10. Collin A., Marzec M., Janiak A., **Sybilska E.**, Jalakas P., Milanowski K., Urban K., Zbieszczyk J., Daszkowska-Golec A. Barley drought-tolerant mutants in the new clothes – the introgression lines for tilling mutants in elite varieties background exposed to drought stress. 13.06-17.06.2022, Embo workshop Molecular responses of plants facing climate change, Montpellier, Francja, współautor prezentacji ustnej (autor prezentujący Anna Collin)
11. Daszkowska-Golec A., Matkowski H., Collin A., **Sybilska E.**, Krol O., Biantari A. ABA as a vaccine for better drought tolerance? - barley physiological and transcriptome response to drought at the pre-flowering stage. 13.06-17.06.2022, Embo workshop Molecular responses of plants facing climate change, Montpellier, Francja, współautor posteru naukowego (autor prezentujący Agata Daszkowska-Golec)
12. Jalakas P., Melzer M., Collin A., **Sybilska E.**, Marzec M., Daszkowska-Golec A. Exploring the impact of drought stress on barley *hvera1* mutants phenotype. 13.06-17.06.2022, Embo workshop Molecular responses of plants facing climate change, Montpellier, Francja, współautor posteru naukowego (autor prezentujący Agata Daszkowska-Golec)
13. **Sybilska E**., Daszkowska-Golec A., Deciphering the Role of Cap-Binding Complex in ABA-Mediated Seed Germination in Barley. 11-17 January 2024, XXXI Plant and Animal Genome Conference, San Diego, California, USA; współautor prezentacji ustnej (autor prezentujący Agata Daszkowska-Golec)



141:1020619512

**14.4 Działania popularyzujące naukę**

1. Noc Biologów, XIII edycja, 26.01.2024, warsztaty: „Odkryj sekrety życia na ziemi - quiz milionerzy". Uniwersytet Śląski w Katowicach.
2. Noc Biologów, XIV edycja, 10.01.2025, warsztaty: „KlimaQuiz: Zagraj o Przyszłość". Uniwersytet Śląski w Katowicach.
3. Śląski Festiwal Nauki, VIII edycja, 07.12-09.12.2024, wykład: „Teraźniejszość medycyny – w stronę spersonalizowanej opieki zdrowotnej". Międzynarodowe Centrum Kongresowe w Katowicach.
4. School of Genomics and New Genomic Techniques (NGT), 18.11-21.11.2024, warsztaty laboratoryjne: „Fundamentals of Plant Genomics and Its Importance in Understanding Plant Response to Climate Change". Uniwersytet Śląski w Katowicach.
5. IAEA Regional Training Course on Molecular Markers and TILLING Applications for Crops Improvement, 25.09-06.10.2023, warsztaty laboratoryjne. Uniwersytet Śląski w Katowicach.
6. Śląski Festiwal Nauki, VII edycja, 09.11-11.12.2023, wykład: „Medycyna spersonalizowana - czy można przygotować terapię „szytą na miarę"?". Międzynarodowe Centrum Kongresowe w Katowicach.
7. Noc Biologów, XII edycja, 13.01.2023, warsztaty: „Zabawa z DNA i szklanką wody". Uniwersytet Śląski w Katowicach.
8. Noc Biologów, XI edycja & Fascination of Plants Day, 18.05.2022, pokaz: Gra „Odkoduj wiadomość". Uniwersytet Śląski w Katowicach.
9. Śląski Festiwal Nauki, VI edycja, 03.12-05.12.2022, warsztat: „Czy kuchnia może zastąpić laboratorium? Wyizoluj DNA z owoców!". Międzynarodowe Centrum Kongresowe w Katowicach.



142:7855370318

**14.5 Nagrody i wyróżnienia**

1. Zwyciężczyni w konkursie „Irene Manton Award Poster" za najlepszy poster naukowy w sekcji roślinnej podczas międzynarodowej konferencji „SEB Centenary Conference 2024" w Pradze, 02.07-05.07.2024, poster naukowy pt. „The role of Cap-Binding Complex (CBC) in ABA-dependent seed germination in barley".
2. Finalistka 6. śląskiej edycji konkursu „Three Minute Thesis" organizowanego przez Politechnikę Śląską w Gliwicach, 17.11.2023, wystąpienie pt. „Zagadki genetyczne kiełkowania: Jak geny wpływają na przetrwanie roślin w trudnych warunkach ?".
3. Wyróżnienie za poster naukowy pt. „Molekularne podstawy odpowiedzi podwójnego mutanta *hvcbp20.ab/hvcbp80.b* na ABA podczas kiełkowania nasion u jęczmienia jarego (*Hordeum vulgare*)" podczas „Dni Młodego Naukowca", 11.09.2023-11.10.2023, w Instytucie Hodowli i Aklimatyzacji Roślin – Państwowym Instytucie Badawczym w Radzikowie.
4. Stypendium projakościowe na rok akademicki 2023/2024, Szkoła Doktorska, Uniwersytet Śląski w Katowicach.

**14.6 Staże i szkolenia**

1. Szkolenie z techniki Visium Spatial Transcriptomics (10x Genomics) w zespole prof. dr. hab. n. med. Andrzeja Pławskiego, 05.08-08.08.2024, Innowacyjne Centrum Medyczne przy Instytucie Genetyki Człowieka PAN w Poznaniu, Polska.
2. Staż naukowy w zespole badawczym „Domestication Genomics" pod kierunkiem dr. Martina Maschera, 08.11.2022 - 25.11.2022, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Niemcy.
3. Kurs „3D RNA-seq – A flexible and powerful tool for differential expression and alternative splicing analysis of RNA-seq data for biologists", 09.11-10.11.2022, Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Niemcy.
4. Kurs „Analiza i wizualizacja danych biologicznych w języku R", 21.10-23.10.2022. data2biology sp. z o.o. online.
5. Kurs „Wprowadzenie do analizy danych RNA-seq", 07.11-08.11.2020, Ideas4biology sp. z o.o. online.



143:9207144986