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Poznanie szlaków transdukcji sygnału strigolaktonów u jęczmienia

ROZPRAWA DOKTORSKA

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W pierwszej kolejności chciałabym podziękować
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które towarzyszyły mi na każdym etapie pracy.
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i zaufanie, jakim mnie obdarzył.

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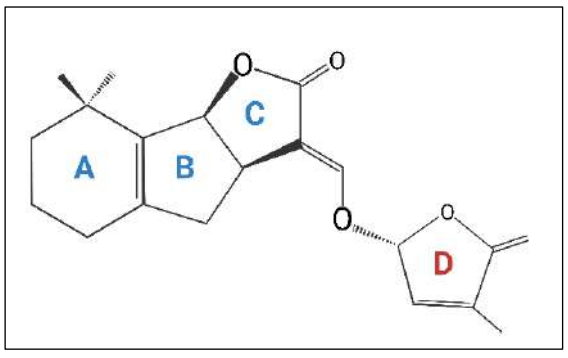
Spis treści

ROZDZIAŁ I

Autoreferat rozprawy

Wprowadzenie

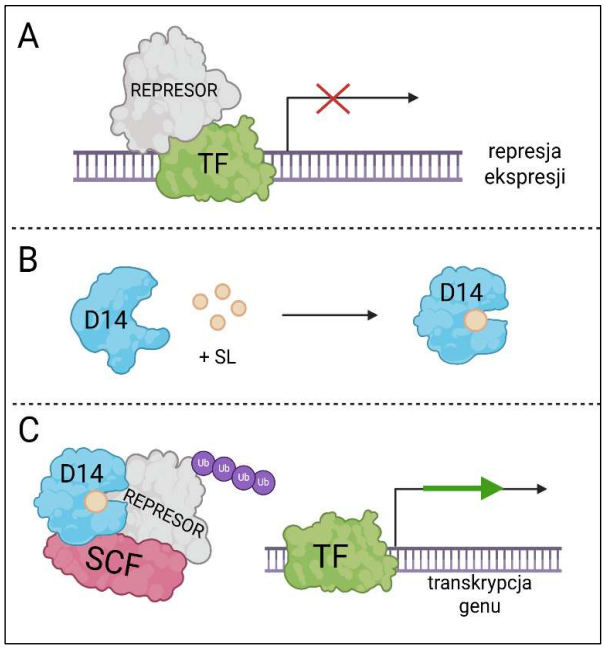
Strigolaktony (SL) to najmłodsza klasa hormonów roślinnych, początkowo opisanych jako cząsteczki sygnałowe indukujące kiełkowanie nasion roślin pasożytniczych (Cook et al., 1966). Do tej pory zidentyfikowano ponad 40 naturalnie występujących SL, które pełnią różnorodne funkcje w kontroli wzrostu i rozwoju roślin (Zhou et al., 2025). Na podstawie struktury chemicznej SL podzielono na kanoniczne i niekanoniczne, w zależności od budowy charakterystycznego trójpierścieniowego rdzenia cząsteczki. W strukturze chemicznej kanonicznych SL wyróżnić można trójcykliczną część laktonową (pierścienie A, B, C) połączoną, za pośrednictwem mostka enolowo-eterowego z butenolidowym pierścieniem laktonowym (pierścień D) (Rycina 1) (Guercio et al., 2023). Pierścienie A, B i C mogą różnić się obecnością dodatkowych grup funkcyjnych (np. $-CH_3$, $-OH$, $-C(O)CH_3$), podczas gdy pierścień D jest silnie konserwatywny i odgrywa kluczową rolę w aktywności biologicznej cząsteczki (Flematti et al., 2016). Kanoniczne SL dzielą się dalej na typy strigolowe i orobancholowe, w zależności od stereochemii pierścienia C, który może przyjmować odpowiednio orientację β- lub α. W przeciwieństwie do kanonicznych SL, niekanoniczne SL wykazują dużą zmienność strukturalną w obrębie części odpowiadającej pierścieniom A, B i C, która często przyjmuje formę otwartą (Ćavar et al., 2015). Niezmiennie jednak niekanoniczne SL również zawierają mostek enolowo-eterowy oraz zachowany pierścień D, kluczowy dla aktywności biologicznej SL.



Rycina 1. Struktura cząsteczki SL z grupy strigoli o orientacji β pierścienia C.

Główne elementy szlaku transdukcji sygnału SL zostały dobrze poznane u wielu gatunków roślin uprawnych oraz gatunków modelowych wykorzystywanych w genetyce roślin, w tym u rzodkiewnika pospolitego (*Arabidopsis thaliana;* Arabidopsis) i ryżu (*Oryza sativa*) (Flematti et al., 2016; Korek i Marzec, 2024. In Strigolactones - Synthesis,

Application and Role in Plants. Academic Press, pp. 53-73). Inicjacja szlaku sygnalizacji SL zachodzi w wyniku rozpoznania i związania cząsteczki SL przez białko receptorowe DWARF14 (D14), należące do rodziny α/β hydrolaz (Hamiaux et al., 2012; Zhao et al., 2013) (Rycina 2). Obecnie jest to jedyny poznany receptor SL u roślin, z wyjątkiem 11 białek z rodziny HYPOSENSITIVE TO LIGHT (HTL) zdolnych do związania cząsteczki SL, zidentyfikowanych u pasożytniczego gatunku Striga (*Striga hermonthica*) (Toh et al., 2015; Yoneyama i Brewer, 2021). Funkcjonalne białko D14 posiada wysoce konserwatywną triadę katalityczną (utworzoną przez reszty Ser96, His246, Asp217), która jest kluczowa dla hydrolizy SL i regulacji ilości bioaktywnych cząsteczek w komórce (Hamiaux et al., 2012; Seto et al., 2019). Badania krystalograficzne wykazały, że pierścień D cząsteczki SL zostaje uwięziony w kieszeni wiążącej D14, co prowadzi do zmiany struktury przestrzennej białka receptora (Zhao et al., 2013). Zmiana ta umożliwia następnie interakcję D14 z pozostałymi białkami wchodzącymi w szlak sygnalizacji SL. Dodatkowo, związanie SL przez D14 destabilizuje receptor, prowadząc do jego degradacji przez ubikwitynację (Shabek et al., 2018). Ostatnie badania przeprowadzone na ryżu sugerują także, że fosforylacja receptora D14 może hamować jego ubikwitynację i degradację, co wzmacnia transdukcję sygnału i regulowane przez SL procesy (Hu et al., 2024). Kolejnym białkiem zaangażowanym w transdukcję sygnału SL jest białko F-box, które oddziałuje z D14 po rozpoznaniu cząsteczki SL. Białko F-box jest podjednostką kompleksu SCF (SKP1-CULLIN-F-BOX), który ubikwitynuje represory transkrypcyjne, kierując je na drogę degradacji proteasomalnej (Zhou et al., 2013). Degradacja represorów SL uwalnia czynniki transkrypcyjne kontrolujące



Rycina 2. Szlak sygnalizacji strigolaktonu (SL). (A) Ekspresja genów zależnych od SL jest hamowana poprzez interakcję represora z czynnikiem transkrypcyjnym (TF). (B) Związanie cząsteczki SL z receptorem DWARF14 (D14) zmienia jego konformację przestrzenną umożliwiając wejście w interakcję z pozostałymi komponentami szlaku sygnalizacji SL. (C) Zmodyfikowany receptor łączy się z białkiem F-box z kompleksu SCF (SKP1-CULLIN-F-BOX). Następnie białko represorowe zostaje zrekrutowane i naznaczone poprzez ubikwitynację do degradacji przez proteasom 26S, co uwalnia TF i umożliwia transkrypcję genów odpowiedzi na SL.

ekspresję genów zależnych od SL. U ryżu i Arabidopsis represory SL kodowane są odpowiednio przez geny *DWARF53* (*D53*) oraz *SUPPRESSOR OF MAX2-LIKE 6* (*SMXL6*), *SMXL7* i *SMXL8* (Jiang et al., 2013; Zhou et al., 2013; Wang et al., 2015). Zarówno D53 jak i SMXL6,7,8, zawierają motyw 'EAR' (ethylene-responsive element binding factor-associated amphiphilic repression, DLNxxP), który bierze udział w represji transkrypcyjnej u roślin (Kagale i Rozwadowski, 2011) oraz motyw 'RGKT', charakterystyczny dla represorów SL (Zhao et al., 2013; Soundappan et al., 2015; Kerr et al., 2021). Motyw represyjny typu 'EAR' jest niezbędny do interakcji z białkiem TOPLESS lub jego homologami, pozwalając na ich oligomeryzację i tworzenie kompleksu represor-korepresor-nukleosom, co fizycznie blokuje inicjację transkrypcji (Jiang et al., 2013; Mach, 2015). Natomiast motyw 'RGKT' jest zaangażowany w interakcje z innymi komponentami szlaku SL. Wykazano na przykładzie SMXL7 oraz D53, że mutacja w obrębie motywu 'RGKT' zapobiega degradacji represora, a przez to trwałym hamowaniem transkrypcji genów zależnych od SL, niezależnie od obecności cząsteczek hormonu w komórce (Zhou et al., 2013; Soundappan et al., 2015). Jedną z trudności w badaniach sygnalizacji SL jest obecność karrikin, ponieważ *MORE AXILLARY GROWTH2* (*MAX2*) oraz *DWARF3* (*D3*) kodujące białka F-box odpowiednio u Arabidopsis i ryżu, stanowią wspólny punkt szlaków sygnalizacyjnych SL i karrikin (Nelson et al., 2011). Z tego względu analizy przeprowadzane na mutantach genów kodujących białko F-box z kompleksu SCF (AtMAX2/OsD3) w kontekście funkcjonowania szlaku sygnalizacji SL wydają się kontrowersyjne. Wykazano, że mutanty *max2* są niewrażliwe zarówno na działanie SL, jak i karrikin, a ich fenotyp może wynikać z zaburzeń w obu szlakach sygnalizacji, podczas gdy mutanty receptorowe: *d14* i *kai2* (*karrikin insensitive2*) wykazują specyficzną niewrażliwość odpowiednio na SL i karrikiny (Smith i Li, 2014; Swarbreck et al., 2020). Ponieważ nie wszystkie komponenty szlaku transdukcji sygnału SL są specyficzne dla tego hormonu, postuluje się, aby to mutanty w genie kodującym receptor SL (D14) były traktowane jako złoty standard w badaniach nad rolą SL w roślinach (Waters et al., 2017).

W 2008 roku po raz pierwszy zaproponowano włączenie SL do grupy fitohormonów na podstawie analiz trzech gatunków roślin, posiadających mutacje w genach kodujących białka zaangażowane w biosyntezę lub sygnalizację SL. U mutantów Arabidopsis (*max3*,

max4), grochu (*Pisum sativum*) (*rms4*, *rms5*; *ramous*) oraz ryżu (*d10*, *d17*, *d3*) zaobserwowano pół-karłowy fenotyp oraz silniejsze, w porównaniu do roślin typu dzikiego, rozkrzewienie pędów (Gomez-Roldan et al., 2008; Umehara et al., 2008). Dalsze badania potwierdziły, że traktowanie roślin syntetycznym analogiem SL - GR24 - przywracało fenotyp roślinom z niedoborem SL, czego nie obserwowano w przypadku mutantów niewrażliwych na SL. W kolejnych latach wykazano również wpływ SL na kształtowanie architektury systemu korzeniowego, zahamowanie wzrostu wtórnego oraz regulację czasu kwitnienia czy senescencji (Agusti et al., 2011; Richmond et al., 2022; Bai et al., 2024). Analogiczne wyniki uzyskano w przypadku mutanta jęczmienia, wyprowadzonego w zespole Genetyki i Genomiki Funkcjonalnej Uniwersytetu Śląskiego w Katowicach metodą mutagenezy chemicznej w połączeniu ze strategią TILLING. U mutanta tego podstawienie guaniny na adeninę w pozycji 725 (G725A) w genie *HvD14* doprowadziło do utraty funkcji kodowanego represora SL (Marzec et al., 2016). Mutacja zlokalizowana w drugim eksonie spowodowała substytucję silnie konserwowanej glicyny na kwas glutaminowy w pozycji 193 (Gly193Glu), która współtworzy tzw. „czapkę helikalną" otaczającą wejście do centrum aktywnego receptora SL (Marzec et al., 2016). W konsekwencji w zmutowanej wersji białka dochodzi do zmniejszenia średnicy wejścia do centrum aktywnego, co fizycznie uniemożliwia związanie cząsteczki SL przez zmutowany receptor (Marzec et al., 2016). Dalsza analiza potwierdziła, że mutant *hvd14.d* wykazuje fenotyp charakterystyczny dla roślin z zaburzoną biosyntezą lub sygnalizacją SL – wytwarza niemal dwukrotnie więcej źdźbeł niż rośliny typu dzikiego (WT) i jest pół-karłem (Rycina 3). Co więcej, traktowanie roślin *hvd14.d* roztworem GR24 o stężeniu 10^{-6} M nie wpłynęło na rozkrzewienie ich części nadziemnej, co jednoznacznie potwierdza niewrażliwość zidentyfikowanego mutanta na działanie SL (Marzec et al., 2016).

Rycina 3. Różnice w wysokości i stopniu rozkrzewienia między rośliną typu dzikiego (genotyp 'Sebastian'), a mutantem *hvd14.d* w fazie kwitnienia. Marzec, M. et al. 2016. Identification and functional analysis of the *HvD14* gene involved in strigolactone signaling in *Hordeum vulgare*. Physiol Plantarum. 158: 341–355; (zmodyfikowano).

Mechanizm szlaku sygnalizacji SL oraz funkcja poszczególnych białek zaangażowanych w przekazywanie tego sygnału wydaje się być dobrze poznana u gatunków modelowych (Waters et al., 2017; Korek i Marzec, 2024. In Strigolactones - Synthesis, Application and Role in Plants. Academic Press, pp. 53-73). Wciąż jednak dysponujemy jedynie podstawowymi informacjami na temat czynników transkrypcyjnych, które regulują odpowiedź rośliny na SL. Pierwszy poznany i dobrze scharakteryzowany czynnik transkrypcyjny zależny od SL - BRANCHED1 (BRC1), należy do rodziny TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1 (TCP) (Wang et al., 2019). Białka należące do tej rodziny posiadają tzw. domenę TCP (motyw helisa-pętla-helisa złożony z 59 aminokwasów), która umożliwia wiązanie się czynników transkrypcyjnych z DNA oraz interakcję typu białko-białko. BRC1 reguluje rozkrzewienie pędów poprzez lokalne działanie w zawiązkach bocznych, gdzie hamuje ich rozwój i przeciwdziała inicjacji nowych odgałęzień pędu (Wang, 2019). Choć brak jest obecnie jednoznacznych dowodów eksperymentalnych na to, że BRC1 jest bezpośrednim celem represora SL, jego ekspresja i aktywność są wyraźnie modulowane przez sygnalizację SL, a zmiany w poziomie transkryptów *BRC1* są ściśle skorelowane z odpowiedzią fenotypową roślin. Rola BRC1 jako elementu szlaku sygnałowego SL została po raz pierwszy opisana u Arabidopsis (Aguilar-Martínez et al., 2007) oraz u grochu (Braun et al., 2012). Wykazano, że zarówno mutanty *atbrc1* oraz *psbrc1* wykazują silnie rozkrzewiony fenotyp, który nie może zostać zahamowany potraktowaniem roślin GR24. Ponadto akumulacja transkryptów *BRC1* jest istotnie zmniejszona zarówno u roślin niewrażliwych na SL, jak i u roślin z mutacją w genach kodujących białka zaangażowane w biosyntezę SL (Aguilar-Martínez et al., 2007; Braun et al., 2012). Kluczowym dowodem potwierdzającym regulację aktywności *BRC1* przez SL jest jego konstytutywna ekspresja u mutantów Arabidopsis pozbawionych funkcjonalnych białek SMXL6/7/8 (Soundappan et al., 2015; Wang et al., 2015; Seale et al., 2017). Do tej pory ekspresja *BRC1* zależna od SL oraz jej homologów u roślin jednoliściennych, TEOSINE BRANCHED1 (TB1), została potwierdzona u wielu gatunków roślin, w tym Arabidopsis, ryżu, grochu, pszenicy (*Triticum aestivum*), kukurydzy (*Zea mays*) i winorośli (*Vitis vinifera*) (Tabela 1).

Tabela 1. Lista homologów genu *BRC1* u różnych gatunków roślin, których ekspresja zależna od strigolaktonu została potwierdzona doświadczalnie.

Gatunek	**Homologi genu *BRC1***	**Referencja**
Arabidopsis thaliana	*BRANCHED1, AtBRC1*	(Aguilar-Martínez et al., 2007)
Oryza sativa	*TEOSINE BRANCHED1, OsTB1*	(Song et al., 2017)
Pisum sativum	*BRANCHED1, PsBRC1*	(Braun et al., 2012)
Triticum aestivum	*TEOSINE BRANCHED1, TaTB1*	(Liu et al., 2017)
Vitis vinifera	*BRANCHED1, VvBRC1*	(Min et al., 2021)
Zea mays	*TEOSINE BRANCHED1, ZmTB1*	(Guan et al., 2012)

SL zostały również opisane jako kluczowy komponent adaptacji roślin do niekorzystnych warunków środowiskowych (Bhatt i Bhatt, 2020). Szczególnie duże zainteresowanie w kontekście regulacji odpowiedzi rośliny na czynniki abiotyczne wzbudza w ostatnich latach współpraca między SL a kwasem abscysynowym - hormonem określanym w literaturze jako hormon stresu (Korek i Marzec, 2023; Singh i Roychoudhury, 2023). Analizy *in silico* wykazały, że elementy *cis*-regulatorowe w promotorach genów biosyntezy SL u Arabidopsis i ryżu znajdują się pod kontrolą czynników transkrypcyjnych związanych z innymi grupami fitohormonów (Marzec i Muszynska, 2015). Większość tych elementów związana jest z czynnikami transkrypcyjnymi zależnymi od kwasu abscysynowego, co wyraźnie podkreśla krzyżowanie się ścieżek sygnalizacji SL i kwasu abscysynowego. Wykazano, że mutanty *d14* Arabidopsis i jęczmienia są bardziej wrażliwe na suszę w porównaniu do WT, co związane jest z wolniejszym zamykaniem szparek, zmienioną ich gęstością na powierzchni liścia oraz cieńszą warstwą kutykuli odkładaną w warunkach stresowych (Li et al., 2020a; Marzec et al., 2020; Daszkowska-Golec et al., 2023). Obniżoną wrażliwość mutanta *hvd14.d* na kwas abscysynowy potwierdzono dodatkowo podczas testu kiełkowania, gdzie zastosowanie 300 μM kwasu abscysynowego ujawniło wyraźną różnicę między analizowanymi genotypami. W przypadku WT kiełkowanie zostało niemal całkowicie zahamowane, podczas gdy *hvd14.d* zachował zdolność kiełkowania na poziomie 73% (Marzec et al., 2020).

Pomimo znacznych postępów w opisaniu funkcji SL w świecie roślin wciąż pozostaje wiele niejasności dotyczących genów i czynników transkrypcyjnych działających

w dalszych etapach szlaku sygnalizacji SL. Identyfikacja nowych czynników transkrypcyjnych zależnych od SL jest kluczowa dla lepszego zrozumienia mechanizmów molekularnych oraz sposobu, w jaki sygnał SL współdziała z innymi szlakami hormonalnymi u roślin. W związku z tym poznanie sieci regulacyjnej czynników transkrypcyjnych zależnych od SL jest kluczowe dla zrozumienia interakcji SL z innymi hormonami w aspekcie wzrostu i rozwoju roślin, a także ich odpowiedzi na czynniki środowiskowe.

Cel prowadzonych badań

Celem prezentowanej rozprawy doktorskiej była identyfikacja molekularnych komponentów szlaku sygnalizacji SL u jęczmienia. W tym celu przeprowadzono analizy fenotypowe, hormonalne i transkryptomiczne z wykorzystaniem mutantów w genie kodującym receptor SL (*HvD14*) oraz represor SL (*HvD53*). Integracja tych danych dla warunków kontrolnych oraz stresu suszy umożliwiła wskazanie genów znajdujących się pod kontrolą SL, a tym samym wytypowanie potencjalnych czynników transkrypcyjnych modulujących odpowiedź roślin w sposób zależny od SL.

Lista powiązanych tematycznie publikacji, wchodzących w skład rozprawy doktorskiej, które stanowią oryginalne rozwiązanie problemu badawczego

1. **Korek M.** i Marzec M. 2023. Strigolactones and abscisic acid interactions affect plant development and response to abiotic stresses. BMC Plant Biology 23: 314

MEiN = 140 pkt, IF_{2024} = 4,8

Publikacja przeglądowa opisująca interakcje pomiędzy szlakami biosyntezy i sygnalizacji SL oraz kwasu abscysynowego zarówno w warunkach kontrolnych, jak i w warunkach stresu abiotycznego.

2. **Korek M**. i Marzec M. 2024. Chapter 4 - An update on strigolactone signaling in plants. In Strigolactones - Synthesis, Application and Role in Plants. Edited by Bashri, G., Hayat, S., and Bajguz, A. pp. 53–73 Academic Press (Elsevier).

MEiN = 50

Rozdział w książce aktualizujący wiedzę z zakresu rozpoznania i wiązania cząsteczek SL przez receptor D14 oraz opisujący szlak transdukcji sygnału SL ze szczególnym

uwzględnieniem zidentyfikowanych do tej pory czynników transkrypcyjnych zależnych od SL.

3. **Korek M**., Uhrig RG., Marzec M. 2025. Strigolactone insensitivity affects differential shoot and root transcriptome in barley. Journal of Applied Genetics 66: 15-28

MEiN = 140, IF_{2024} = 1,9

Publikacja oryginalna opisująca fenotyp oraz zależne od SL zmiany w transkryptomie pędu i korzenia 3-tygodniowych siewek jęczmienia odmiany ‘Sebastian’ oraz mutanta *hvd14.d* rosnących w warunkach kultury hydroponicznej. Analizy transkryptomiczne wykonane osobno dla poszczególnych organów umożliwiły wytypowanie metodami *in silico* czynników transkrypcyjnych potencjalnie zależnych od SL.

4. **Korek M**., Mehta D., Uhrig GR., Daszkowska-Golec A., Novak O., Buchcik W., Marzec M. 2025. Strigolactone insensitivity affects the hormonal homeostasis in barley. Scientific Reports 15: 9375

MEiN = 140, IF_{2024} = 3,9

Publikacja oryginalna przedstawiająca wpływ mutacji w genie *HvD14*, kodującym receptor SL, na architekturę pędu jęczmienia na przestrzeni całego cyklu rozwojowego aż do dojrzałości roślin. Dodatkowo przeprowadzono analizę profilu hormonalnego 2-tygodniowych i 4-tygodniowych siewek odmiany ‘Sebastian’ oraz mutanta *hvd14.d.* Kolejno, analizy transkryptomiczne i proteomiczne umożliwiły selekcję genów i białek zależnych od SL, które uczestniczą w utrzymaniu homeostazy hormonalnej. Z kolei wykorzystanie narzędzi bioinformatycznych pozwoliło na selekcję czynników transkrypcyjnych potencjalnie zależnych od SL.

5. **Korek M**., Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology, pcaf09

MEiN = 140, IF_{2024} = 4,0

Publikacja oryginalna opisująca fenotyp, wydajność procesu fotosyntezy oraz tolerancję na suszę u wyprowadzonego w ramach rozprawy doktorskiej mutanta jęczmienia *hvd53a.f*, posiadającego mutację w genie *HvD53A* kodującym represor SL.

Kolejno analizy transkryptomiczne oraz bioinformatyczne pozwoliły na selekcję czynników transkrypcyjnych potencjalnie zależnych od SL, które mogą wyjaśniać kontrastowy fenotyp mutantów *hvd53a.f* i *hvd14.d* rosnących w warunkach kontrolnych oraz narażonych na stres suszy.

Suma punktów MEiN = 610, Suma IF = 14,6

Materiały i metody

Materiał roślinny

Materiał badawczy wykorzystany w niniejszej rozprawie doktorskiej stanowiły rośliny jęczmienia zwyczajnego (*Hordeum vulgare* L.):

- odmiana 'Sebastian' stanowiąca genotyp kontrolny (typ dziki, WT), która jest odmianą wyjściową dla populacji HorTILLUS, utworzonej w Zespole Genetyki i Genomiki Funkcjonalnej Roślin (ZGiGFR) na Uniwersytecie Śląskim w Katowicach, z wykorzystaniem mutagenów chemicznych: azydku sodu (NaN_3) oraz N-metylonitrozomoczniku (MNU) (Szurman-Zubrzycka et al., 2018)

- mutant *hvd14.d*, zidentyfikowany z wykorzystaniem strategii TILLING w obrębie populacji HorTILLUS, wyprowadzony w ramach wcześniej prowadzonych prac badawczych w zespole ZGiGFR, posiadający recesywną mutację (G725A, Gly193Glu) w drugim eksonie genu *HvD14* kodującym receptor SL (Marzec et al., 2016)

- mutant *hvd53a.f*, zidentyfikowany z wykorzystaniem strategii TILLING w obrębie populacji HorTILLUS, wyprowadzony w ramach przedstawionej rozprawy doktorskiej, posiadający recesywną mutację (T4001C, Ser664Pro) w trzecim eksonie genu *HvD53A* kodującym represor SL

Izolacja materiału genetycznego i analiza transkryptomu

W każdym z badań RNA izolowano w czterech powtórzeniach biologicznych; materiał genetyczny izolowano z tkanek roślinnych przy użyciu zestawu mirVana miRNA Isolation Kit (Thermo Fisher Scientific, numer katalogowy: AM1560). Konstrukcję bibliotek oraz sekwencjonowanie (odczyty parowane, 150 nukleotydów) na platformie Illumina NovaSeq™ 6000 przeprowadzono w Novogene Genomics Service (Cambridge,

Wielka Brytania). Analiza danych RNA-seq przebiegała w następujących etapach. W pierwszym kroku sprawdzono jakość uzyskanych sekwencji i usunięto odczyty niskiej jakości, aby zapewnić wiarygodność dalszych analiz. Następnie wysokiej jakości sekwencje dopasowano do genomu referencyjnego w celu identyfikacji poszczególnych transkryptów. Kolejnym etapem była ocena poziomu ekspresji genów, co umożliwiło wytypowanie genów o zróżnicowanych poziomach ekspresji między porównywanymi grupami (wartość $p < 0,05$, a $\log2FC \geq 1$ lub ≤ -1).

Analiza sekwencji promotorowych i identyfikacja czynników transkrypcyjnych potencjalnie zależnych od SL

Do analizy sekwencji promotorowych pobrano 1500 par zasad znajdujących się przed kodonem START (opcja „Flank Gene") genów o zróżnicowanej ekspresji, korzystając z narzędzia BioMart (https://plants.ensembl.org/index.html) oraz zestawu danych *Hordeum vulgare* genes (Morex_V2_scaf lub IBSC_v2). Uzyskane pliki wykorzystano jako dane wejściowe do identyfikacji potencjalnych interakcji regulacyjnych pomiędzy czynnikami transkrypcyjnymi a motywami *cis*-regulatorowymi obecnymi w sekwencjach promotorowych, przy użyciu narzędzia PlantRegMap – Regulatory prediction (https://plantregmap.gao-lab.org/). Równolegle przeprowadzono analizę mającą na celu wyłonienie czynników transkrypcyjnych, których targety są nad-reprezentowane w analizowanym zbiorze genów. Homologi Arabidopsis zidentyfikowanych czynników transkrypcyjnych u jęczmienia wyselekcjonowano przy użyciu bazy danych Plant Transcription Factor Database (https://planttfdb.gao-lab.org/).

Wykorzystane w rozprawie doktorskiej metody mające na celu opis mutantów *hvd14.d* i *hvd53a.f* skupiają się przede wszystkim na analizach transkryptomicznych oraz identyfikacji nowych czynników transkrypcyjnych potencjalnie zaangażowanych w sygnalizację SL u jęczmienia. Pozostałe techniki badawcze wykorzystane w prowadzonych badaniach zostały szczegółowo opisane w pracach Korek et al., 2024, 2025a, 2025b przedstawionych w rozprawie, odpowiednio jako rozdziały III.3, III.4 III.5.

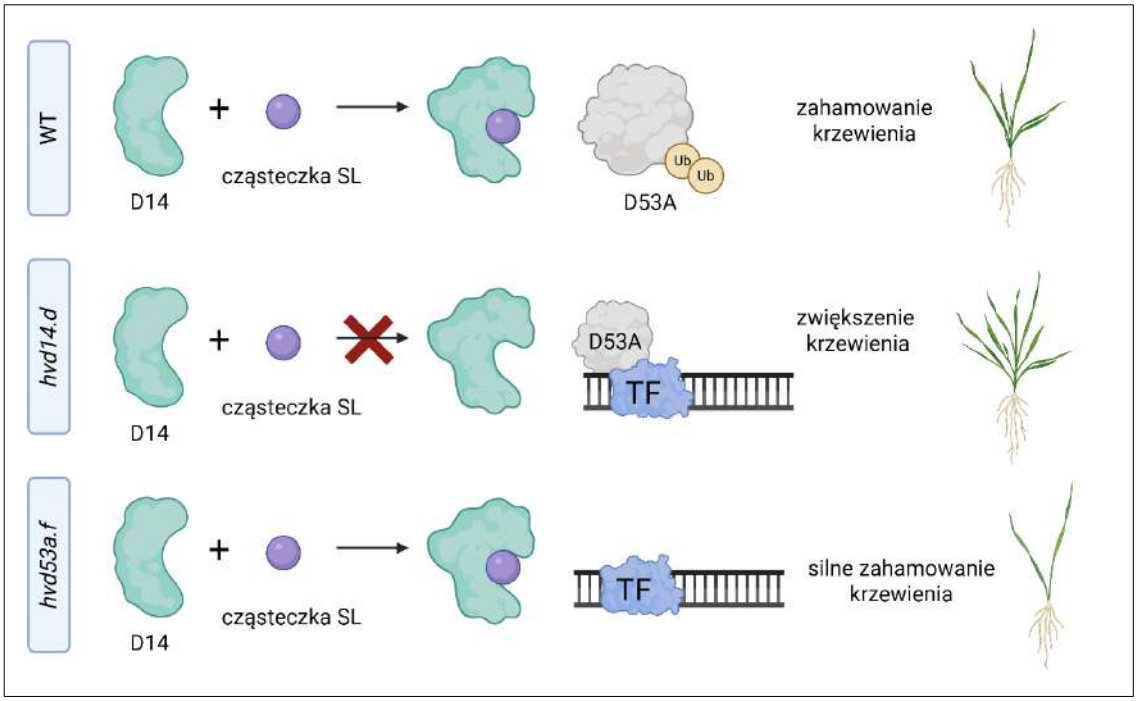
Wyniki i dyskusja

Rola sygnalizacji SL w regulacji rozkrzewiania jęczmienia

Rozkrzewianie, czyli zdolność roślin do wytwarzania bocznych pędów, stanowi jedną z kluczowych cech agronomicznych, wpływających bezpośrednio na architekturę roślin,

liczbę organów generatywnych, a w konsekwencji na wielkość uzyskiwanego plonu (Barbier et al., 2019).

W niniejszych badaniach przeprowadzono analizę fenotypową odmiany wyjściowej jęczmienia ‘Sebastian’ oraz mutantów wyprowadzonych metodą mutagenezy chemicznej i strategią TILLING, które niosły mutacje w genach *DWARF14* (G725A, Gly193Glu) i *DWARF53A* (T4001C, Ser664Pro), kodujących odpowiednio receptor i represor szlaku sygnalizacji SL (Rycina 4). Analizowane genotypy porównano w dwóch systemach uprawy - w glebie, stanowiącej standardowe warunki wzrostu roślin (Korek et al., 2025b, 2025a) oraz w kulturze hydroponicznej, zapewniającej stały dostęp do wody i składników odżywczych (Korek et al., 2024).



Rycina 4. Mutanty jęczmienia *hvd14.d* i *hvd53a.f* wykazują przeciwstawne fenotypy w zakresie rozkrzewiania. Mutacja w genie *HvDWARF14* (*HvD14*) prowadzi do utraty zdolności wiązania strigolaktonów (SL), co uniemożliwia degradację represora sygnalizacji SL i skutkuje zahamowaniem represji transkrypcyjnej genów zależnych od SL. W efekcie obserwuje się znaczne zwiększenie liczby pędów bocznych. Odwrotny efekt powoduje mutacja w genie *HvD53A*, kodującym represor SL — jej obecność prowadzi do zniesienia regulacji negatywnej, co skutkuje konstytutywną represją genów zależnych od SL i silnym ograniczeniem rozkrzewiania; TF – czynnik transkrypcyjny.

Jak wcześniej wykazano, półkarłowy mutant jęczmienia *hvd14.d* wytwarza prawie dwukrotnie więcej pędów bocznych niż WT, gdy rośliny były uprawiane w glebie (Marzec et al., 2016). Podobne wyniki uzyskano w pracach badawczych wchodzących w skład prezentowanej rozprawy doktorskiej. Dojrzałe rośliny *hvd14.d* rozwinęły prawie 50% więcej źdźbeł w porównaniu do odmiany wyjściowej ‘Sebastian’ (odpowiednio

27 ± 4,9 i 14 ± 3,3), przy czym różnice w architekturze pędu stawały się widoczne i statystycznie istotne u 4-tygodniowych roślin (Korek et al., 2025b). Z kolei kultura hydroponiczna 3-tygodniowych siewek jęczmienia w pożywce ½ Hoaglanda (Hothem et al., 2003) zwiększyła liczbę pędów bocznych mutanta *hvd14.d* oraz WT w porównaniu z warunkami glebowymi. Jednakże różnice między analizowanymi genotypami utrzymywały się na podobnym poziomie – u *hvd14.d* liczba źdźbeł była o 60% większa w niż u WT (odpowiednio 5,1 ± 0,68 i 3,1 ± 0,61) (Korek et al., 2024). W literaturze wykazano, że warunki kultury hydroponicznej sprzyjają rozwojowi pędów w porównaniu do uprawy w glebie, co może wynikać z łatwego dostępu do wody i składników odżywczych (Dutta et al., 2023). Dodatkowo, wysokość mutanta *hvd14.d* rosnącego zarówno w glebie, jak i w warunkach hydroponicznych była obniżona o około 20% względem odmiany wyjściowej 'Sebastian'. Uzyskane wyniki wskazują, że mutacja *hvd14.d* prowadzi do półkarłowego wzrostu oraz zwiększonego rozkrzewiania niezależnie od warunków uprawy, a efekt ten utrzymuje się przez cały cykl rozwojowy rośliny, co potwierdza kluczową rolę sygnalizacji SL i receptora HvD14 w regulacji architektury pędu jęczmienia. Co, więcej udział SL w mechanizmie rozkrzewiania jęczmienia potwierdzono także, przeprowadzając analizę fenotypową mutanta *hvd53a.f,* wyprowadzonego w ramach niniejszej rozprawy doktorskiej (Rycina 4). Ponieważ mutacje w genach kodujących komponenty sygnalizacji SL prowadzą do zwiększenia ilości produkowanych źdźbeł, postawiono hipotezę, iż mutacja w genie kodujących represor SL, może wywoływać odwrotny efekt, ze względu na konstytutywną aktywację szlaku sygnalizacji SL. Statystycznie istotne zmniejszenie rozkrzewiania było widoczne już u 3-tygodniowych roślin jęczmienia niosących mutację w genie *HvD53A* (T4001C, Ser664Pro) w porównaniu do WT i utrzymywało się przez cały okres rozwoju roślin. Dla dojrzałych roślin mutanta *hvd53a.f* wykazano 30% zmniejszenie liczby pędów bocznych, w porównaniu do odmiany 'Sebastian' (mutant: 11 ± 1,5, WT: 16 ± 1,4) (Korek et al., 2025a). Ponadto mutant *hvd53a.f* cechował się zwiększoną wysokością roślin o 14% w porównaniu do WT (WT: 65,2 ± 2,73 cm, mutant: 74,5 ± 3,44 cm). Przedstawione analizy potwierdzają przeciwstawną rolę HvD14 i HvD53A w regulacji architektury pędu u jęczmienia. Zwiększona liczba pędów bocznych u mutanta *hvd14.d* oraz ich redukcja u mutanta *hvd53a.f* wskazują, że zarówno percepcja, jak i represja sygnału SL wpływają na rozkrzewienie jęczmienia. Ponadto wykazano, że mutacja *hvd53a.f* powoduje 42% redukcję zawartości chlorofilu (WT: 32.4 ± 3.2 a.u.;

mutant: 18.9 ± 1.78 a.u.) oraz opóźnia kwitnienie o 19 dni w porównaniu z odmianą wyjściową ‘Sebastian’ (Korek et al., 2025a).

Wpływ mutacji w genie HvD14 na profil hormonalny jęczmienia

Hormony roślinne funkcjonują w ramach złożonych sieci regulacyjnych, w których poszczególne szlaki biosyntezy i sygnalizacji wzajemnie na siebie oddziałują, umożliwiając kontrolę wzrostu i rozwoju roślin oraz ich odpowiedź na stresowe czynniki środowiskowe (Jaillais i Chory, 2010). Współdziałanie sieci hormonalnej potwierdzają analizy *in silico* przeprowadzone u Arabidopsis, które wykazały ponad 2000 potencjalnych interakcji typu białko–białko w obrębie szlaków sygnalizacji i biosyntezy fitohormonów (Altmann et al., 2020). Wykorzystując mutanta jęczmienia *hvd14.d* oraz jego odmianę rodzicielską 'Sebastian', przeprowadzono profilowanie zawartości fitohormonów w tkankach pędu obu genotypów (Korek et al., 2025b). Analizy wykonano dla roślin w wieku 2 i 4 tygodni – odpowiednio na etapie, gdy nie zaobserwowano jeszcze różnic w rozkrzewieniu oraz gdy zaczynają się one uwidaczniać w sposób statystycznie istotny.

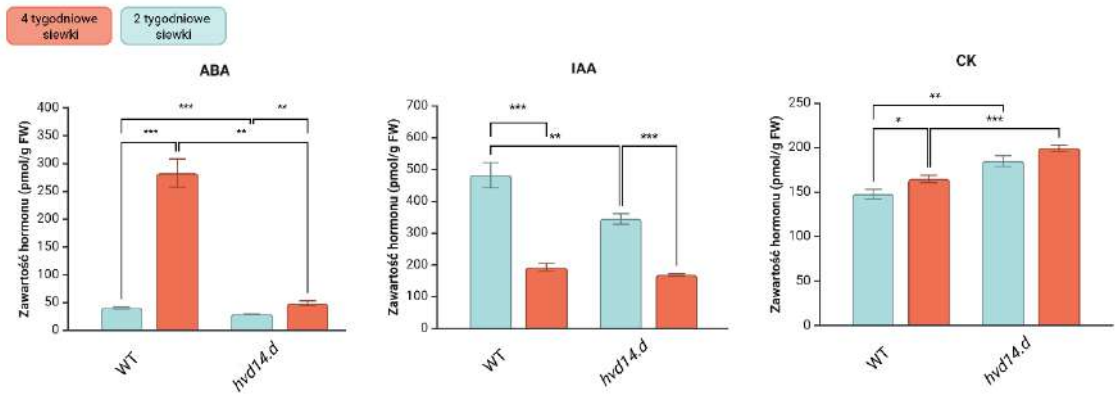
Największe zmiany w zawartości fitohormonów między WT a mutantem *hvd14.d* dotyczą kwasu abscysynowego, zarówno u 2-tygodniowych, jak i 4-tygodniowych siewek jęczmienia (Rycina 5). Brak funkcjonalnego białka HvD14 prowadził do obniżenia poziomu kwasu abscysynowego w pędach mutanta w porównaniu z WT, niezależnie od wieku roślin. Różnice te są jednak znacznie wyraźniejsze u starszych roślin, co może wiązać się z ich bardziej zaawansowanym stopniem rozwoju. U Arabidopsis, w celu określenia miejsca akumulacji kwasu abscysynowego w optymalnych warunkach wzrostu, pęd rośliny rozdzielono na łodygę, młode liście, młode kwiaty, wierzchołek pędu głównego i zawiązki boczne (Yao i Finlayson, 2015). Najwyższe stężenia kwasu abscysynowego odnotowano w tkankach merystematycznych pędu, co wskazuje na udział kwasu abscysynowego w podziałach komórkowych i inicjacji nowych organów. Zgodnie z tym, silnie rozgałęzione mutanty, takie jak Arabidopsis *max2* i *brc1*, wykazują obniżoną zawartość kwasu abscysynowego w zawiązkach bocznych (Yao i Finlayson, 2015). Dodatkowo wykazano, że BRC1 wiąże się z i pozytywnie reguluje ekspresję *HOMEOBOX PROTEIN 21* (HB21), *HB40* i *HB53,* które zwiększają transkrypcję *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3*), kluczowego enzymu w szlaku biosyntezy kwasu abscysynowego (González-Grandío et

al., 2017). W związku z tym, mutacja w genie *HvD14* może skutkować obniżeniem poziomu kwasu abscysynowego w pędzie jęczmienia, a tym samym regulować fenotyp mutanta *hvd14.d* (Korek et al., 2025b).

Różnice w koncentracji hormonów między WT i *hvd14.d* zaobserwowano także w przypadku auksyny i cytokinin, kluczowych regulatorów rozkrzewiania pędów (Rycina 5) (Shimizu-Sato et al., 2009). Model interakcji SL-auksyna w pędzie roślin opisuje wpływ SL na polarną lokalizację transporterów auksyny z rodziny PIN-FORMED (PIN) (Shinohara et al., 2013). Wykazano, iż SL indukują szybkie usuwanie białek PIN z błony plazmatycznej komórek miękiszu ksylemu w łodydze, prowadząc do zaburzenia kierunkowego transportu auksyny. W efekcie, poprzez ograniczenie możliwości eksportu auksyny z komórek merystematycznych, SL mogą w sposób dynamiczny i odwracalny modulować system kanalizacji auksyny w pędzie, a tym samym regulować intensywność rozkrzewiania roślin, w zależności od stanu fizjologicznego i warunków środowiskowych (Nahas et al., 2024). U 4-tygodnowych siewek *hvd14.d* nie stwierdzono istotnych różnic w poziomie auksyny w porównaniu do WT, co może wynikać z zaburzeń w jej transporcie w obrębie pędu, a nie zmian w biosyntezie (Korek et al., 2025b). Jednakże, zaobserwowany wyraźny spadek auksyny pomiędzy 4-tygodniowymi a 2-tygodnowymi siewkami obu genotypów, wskazuje na możliwe ograniczenia w biosyntezie/akumulacji auksyny na przestrzeni rozwoju siewki. Podczas gdy, SL i auksyna współdziałają hamując rozkrzewianie pędu, wzrost stężenia cytokinin wywołuje efekt przeciwny (Barbier et al., 2019). U ryżu oraz grochu wykazano, że poziom transkryptów *BRC1* obniża się w sposób zależny od dawki cytokinin, co prowadzi jednocześnie do zwiększenia rozkrzewiania rośliny (Braun et al., 2012; Dierck et al., 2016). Ponadto, traktowanie siewek ryżu auksyną prowadzi do obniżenia ekspresji genów biosyntezy cytokinin oraz zwiększenia ekspresji genów biosyntezy i sygnalizacji SL w pędzie, podkreślając kluczową rolę sieci cytokininy-auksyna-SL w regulacji procesu krzewienia (Aguilar-Martínez et al., 2007; Wang, 2019; Xu et al., 2015). Zarówno u 2-tygodniowych, jak i 4-tygodniowych siewek *hvd14.d* zaobserwowano podwyższony poziom cytokinin w porównaniu do mniej rozkrzewionego WT (Rycina 5). Zgromadzone dane wskazują, że równowaga między poziomami SL, auksyną i cytokininami oraz ich wzajemna regulacja stanowią kluczowy mechanizm kontroli rozwoju pędów roślin. Interakcja tych fitohormonów odgrywa zatem zasadniczą rolę w determinowaniu stopnia rozkrzewienia

u jęczmienia, prawdopodobnie poprzez integrację sygnałów hormonalnych z regulacją ekspresji genów takich jak *BRC1*.

Mutacja w genie *HvD14* wpłynęła także na zawartość kwasu jasmonowego oraz kwasu salicylowego w siewkach jęczmienia (Korek et al., 2025b). Oba hormony uczestniczą między innymi w reakcjach obronnych roślin, jednak do tej pory nie wykazano ich bezpośredniej interakcji ze szlakiem biosyntezy czy sygnalizacji SL. Z tego względu zmieniona zawartość kwasu jasmonowego i kwasu salicylowego u mutanta *hvd14.d* może prawdopodobnie wynikać z zaburzenia ogólnej homeostazy hormonalnej.



Rycina 5. Mutacja w genie *HvDWARF14* (*HvD14*) wpływa na homeostazę kwasu abscysynowego (ABA), auksyny (IAA) i cytokinin (CK) u jęczmienia. Gwiazdkami oznaczono statystycznie istotne różnice między próbkami według testu t-Studenta (wartości p odpowiadają: *p < 0,05; **p < 0,01; ***p < 0,001).

Molekularne podstawy różnic rozwojowych u mutanta hvd14.d i hvd53a.f

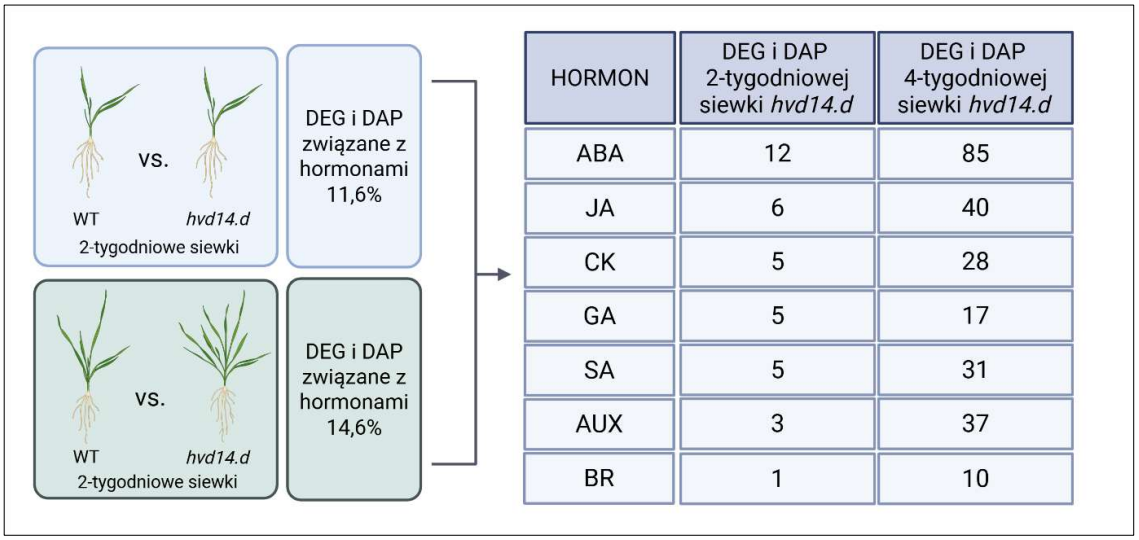
W celu poznania molekularnych podstaw różnic fenotypowych obserwowanych między mutantami *hvd14.d* i *hvd53a.f* a WT, przeprowadzono serię eksperymentów obejmujących analizy transkryptomiczne oraz proteomiczne. W każdym z badań zidentyfikowano liczne geny o zróżnicowanej ekspresji (DEG) lub białka o zmiennym poziomie akumulacji (DAP), co potwierdza szeroki wpływ SL na regulację wzrostu i rozwoju siewek jęczmienia, jak również na odpowiedź roślin na czynniki środowiskowe (Aliche et al., 2020).

Porównanie transkryptomu 3-tygodniowych siewek mutanta *hvd14.d* oraz roślin WT, rosnących w warunkach kultury hydroponicznej, pozwoliło na wyselekcjonowanie ponad 6 000 genów potencjalnie zależnych od SL, które były charakterystyczne dla pędu lub korzenia, bądź wykazywały ekspresję niezależną od rodzaju tkanki (Korek et al., 2024). Blisko 80% DEG zidentyfikowano w tkance korzenia, co może wskazywać na

dominującą aktywność SL w regulacji procesów zachodzących w systemie korzeniowym młodych roślin jęczmienia, w kontraście do rozwijającego się pędu. Należy jednak zaznaczyć, że obserwowana przewaga liczby DEG w korzeniu może również wynikać z większej złożoności strukturalnej tego organu na analizowanym etapie rozwojowym, między innymi poprzez obecność wielu różnych typów komórek, rozwijających się korzeni zarodkowych i setek korzeni bocznych (Shahan et al., 2022). Z kolei pęd rośliny w tym wieku tworzy zwykle trzy do pięciu odgałęzień znajdujących się w fazie wegetatywnej, które nie wykształciły jeszcze międzywęźli. Podobną dysproporcję zaobserwowano w kolejnej pracy badawczej, gdzie porównywano transkryptom siewek 2-tygodniowych i 4-tygodniowych mutanta *hvd14.d* i WT wzrastających w warunkach glebowych (Korek et al., 2025b). Liczba zidentyfikowanych DEG była ponad 10-krotnie wyższa u 4-tygodniowych roślin (2-tygodniowe siewki: 94 DEG, 4 tygodniowe siewki: 1134 DEG), co może wynikać zarówno z bardziej zaawansowanego stadium rozwojowego, jak i obserwowanych różnic fenotypowych w rozkrzewianiu. Co więcej, jedynie 30 DEG było wspólnych dla młodszych i starszych roślin, co wskazuje, że procesy zależne od SL zachodzące podczas rozwoju jęczmienia są dynamiczne i specyficzne dla danego etapu rozwojowego.

W dalszej części analiz funkcjonalna adnotacja DEG oraz DAP różniących mutanta *hvd14.d* i WT wykazała, że zmiany związane z procesami hormonalnymi odpowiadają odpowiednio prawie 12% różnic obserwowanych w siewkach 2-tygodniowych i 15% w siewkach 4-tygodniowych (Korek et al., 2025b) (Rycina 6). Co istotne, udział poszczególnych kategorii hormonów przypisanych do DEG i DAP odzwierciedla zaobserwowane zmiany w poziomach hormonów u mutanta *hvd14.d*, wskazując na ścisły związek między transkryptomem/proteomem a siecią hormonalną. Największe zmiany zarówno w zakresie zawartości hormonów, jak i liczby genów i białek zaangażowanych w procesy regulowane hormonami, dotyczyły kwasu abscysynowego. Zależności pomiędzy SL a kwasem abscysynowym, obejmujące wzajemne oddziaływania szlaków biosyntezy i sygnalizacji w kontekście wzrostu, rozwoju roślin oraz odpowiedzi na stres abiotyczny, zostały szeroko udokumentowane w literaturze (Cheng et al., 2017; Korek i Marzec, 2023). Dodatkowo wcześniejsze badania nad mutantem *hvd14.d* wykazały jego mniejszą wrażliwość na kwas abscysynowy w teście kiełkowania ziarniaków (Marzec et al., 2020). Co więcej, wykazano że zależny od SL czynnik transkrypcyjny BRC1, reguluje lokalnie w zawiązkach bocznych transkrypcję dwóch

genów związanych w sygnalizacją kwasu abscysynowego: *ABA-RESPONSIVE ELEMENT BINDING FACTOR 3* (*ABF3*) oraz *ABA-INSENSITIVE 5* (*ABI5*), uznawanych za kluczowe elementy tego szlaku (van Es et al., 2024).



HORMON	DEG i DAP 2-tygodniowej siewki *hvd14.d*	DEG i DAP 4-tygodniowej siewki *hvd14.d*
ABA	12	85
JA	6	40
CK	5	28
GA	5	17
SA	5	31
AUX	3	37
BR	1	10

Rycina 6. Zmiany w transkryptomie i proteomie 2-tygodniowych i 4-tygodniowych siewek mutanta *hvd14.d* związane z niewrażliwością na strigolakton. Tabela przedstawia liczbę genów o zróżnicowanej ekspresji (DEG) i białek o zmiennym poziomie akumulacji (DAP) zaangażowanych w procesy związane z fitohormonami. ABA – kwas abscysynowy, JA – kwas jasmonowy, CK – cytokininy, GA – gibereliny, SA – kwas salicylowy, AUX – auksyny, BR – brasinosteroidy.

Kolejno, przeprowadzono analizę transkryptomiczną wyprowadzonego w ramach rozprawy doktorskiej mutanta *hvd53a.f* (Korek et al., 2025a). Uzyskane wyniki ujawniły 4342 DEG, wskazując na szeroką skalę zmian w transkryptomie związanych z zaburzeniem sygnalizacji SL. Analiza nad-reprezentacji terminów GO (ang. gene ontology enrichment) przeprowadzona dla genów o podwyższonej ekspresji ujawniła istotne powiązania z metabolizmem oksylipin oraz glutationu. Oksylipiny, powstające w wyniku utleniania kwasów tłuszczowych, są znane przede wszystkim z udziału w odpowiedzi na stres abiotyczny (Knieper et al., 2023), jednak liczne badania wskazują również na ich zaangażowanie w regulację takich procesów jak kwitnienie, starzenie się liści, rozwój korzeni bocznych czy zamykanie aparatów szparkowych – zarówno w sposób zależny, jak i niezależny od kwasu abscysynowego (Vellosillo et al., 2007; Reinbothe et al., 2009; Montillet et al., 2013; Simeoni et al., 2022). Co istotne, zarówno oksylipiny, jak i glutation pełnią kluczową rolę w utrzymaniu homeostazy redoks w komórkach, a ich wzmożona aktywność może wskazywać na zaangażowanie tych szlaków w odpowiedź na stres oksydacyjny. Spośród dziesięciu genów o najwyższym poziomie zróżnicowanej ekspresji, cztery należały do rodziny *DEHYDRIN* (*HvDHN1–4*), osiągając wartości log2FC w zakresie od 7,65 do 9,21 (Korek et al.,

2025a). Białka kodowane przez te geny znane są ze swojej roli w stabilizacji błon komórkowych, retencji wody oraz detoksykacji ROS, co czyni je istotnym elementem odpowiedzi roślin na stres środowiskowy (Riyazuddin et al., 2022). Zwiększona ekspresja *HvDHN1–4* u roślin *hvd53a.f*, w połączeniu z intensyfikacją metabolizmu oksylipin i glutationu, może zatem świadczyć o ich zwiększonym potencjale adaptacyjnym w warunkach deficytu wody. Wyniki te znajdują dodatkowe potwierdzenie w analizie barwienia DAB, która wykazała najwyższy poziom aktywności antyoksydacyjnej u mutanta *hvd53a.f* w porównaniu z roślinami WT i *hvd14.d*. Obserwacje te wspólnie wskazują, że uruchomienie ścieżek związanych ze zwalczaniem stresu oksydacyjnego może stanowić kluczowy element mechanizmów adaptacyjnych mutanta *hvd53a.f*.

Identyfikacja i funkcjonalna charakterystyka czynników transkrypcyjnych zależnych od SL u jęczmienia

Wykorzystując zestaw narzędzi bioinformatycznych, przeprowadzono analizę nad-reprezentowanych motywów *cis*-regulatorowych obecnych w promotorach wcześniej zidentyfikowanych DEG oraz genów kodujących DAP zależnych od SL (Korek et al., 2024, 2025a, 2025b). Podejście to umożliwiło wytypowanie potencjalnych czynników transkrypcyjnych, zaangażowanych w odpowiedź jęczmienia na SL.

W analizach prowadzonych w warunkach hydroponicznych zidentyfikowano 28 czynników transkrypcyjnych, które mogą uczestniczyć w przekazywaniu sygnału SL, regulując zmiany fenotypowe obserwowane w architekturze pędu i systemu korzeniowego 3-tygodniowych siewek *hvd14.d* (Korek et al., 2024). Co istotne, geny kodujące te czynniki transkrypcyjne należą również do grupy genów o zróżnicowanej ekspresji, co sugeruje, że pełnią one podwójną rolę – jako regulatorzy ekspresji innych genów oraz jako elementy same podlegające regulacji w odpowiedzi na zaburzenia w sygnalizacji SL. Dodatkowo, ponad połowa z tych czynników transkrypcyjnych (18/28, 75%) tworzy sieć znanych i przewidywanych interakcji, sugerując ścisłą współpracę między czynnikami transkrypcyjnymi w regulacji szlaków zależnych od SL u jęczmienia. Największa zidentyfikowana sieć obejmuje 12 białek, w tym siedem należących do rodziny WRKY. Na podstawie adnotacji funkcjonalnej oraz dostępnych danych literaturowych wykazano, że zidentyfikowane czynniki transkrypcyjne biorą

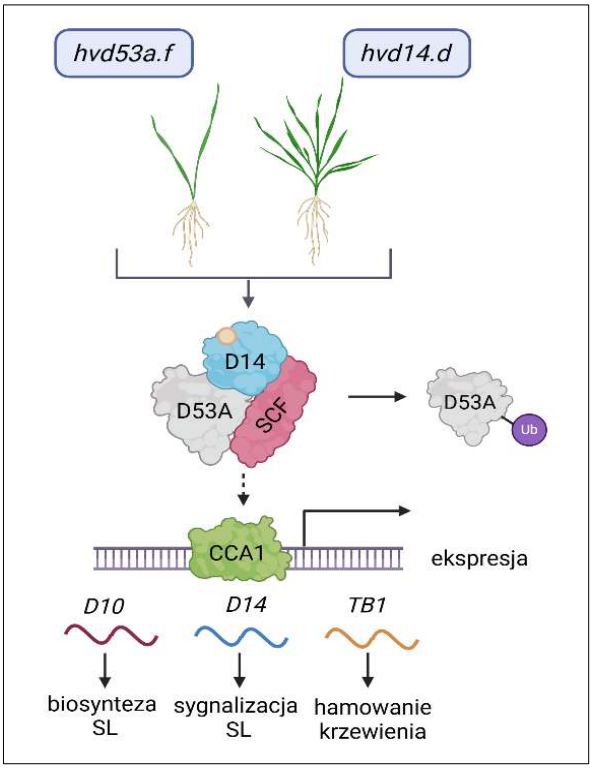
udział m.in. w odpowiedzi na kwas abscysynowy i auksynę, w reakcji na niedobór fosforu, a także w biosyntezie kutyny (Korek et al., 2024).

Analizy transkryptomiczne i proteomiczne przeprowadzone na 2-tygodniowych i 4-tygodniowych siewkach mutanta *hvd14.d* rozwijających się w glebie umożliwiły identyfikację kolejnej grupy potencjalnych czynników transkrypcyjnych zależnych od SL (Korek et al., 2025b). Wśród zidentyfikowanych DEG oraz DAP łącznie rozpoznano 109 czynników transkrypcyjnych, spośród których homologii czterech genów zostały wcześniej opisane u Arabidopsis jako reagujące na sygnał SL (Wang et al., 2020b). Jednym ze zidentyfikowanych czynników transkrypcyjnych jest BRC1 (AT3G18550) szeroko opisywany w literaturze jako kluczowy element w regulacji rozkrzewiania pędów zależnej od SL (Wang et al., 2019). Kolejnym nowo zidentyfikowanym czynnikiem transkrypcyjnym potencjalnie zależnym od SL jest MYB DOMAIN PROTEIN 88 (MYB88, AT2G02820), który działając wspólnie z FOUR LIPS (FLP), reguluje różnicowanie komórek szparkowych oraz architekturę systemu korzeniowego w warunkach stresu suszy (Xie et al., 2010; Sun et al., 2015). Podwójne mutanty *flp/myb88* u Arabidopsis nie wykazują wyraźnych fenotypów w optymalnych warunkach wzrostu, jednak cechują się istotnie zwiększoną podatnością na suszę, wynikającą z nadmiernej liczby aparatów szparkowych rozmieszczonych na powierzchni liścia (Xie et al., 2010). Wcześniejsze badania wykazały, że zwiększona wrażliwość na suszę obserwowana u mutanta *hvd14.d* wiąże się m.in. z wolniejszym zamykaniem aparatów szparkowych oraz ich zmienioną gęstością (Marzec et al., 2020). Wyniki te sugerują, że zaburzona sygnalizacja SL może wpływać na obniżoną ekspresję *MYB88*, co z kolei prowadzi do nadmiernego różnicowania komórek szparkowych i w konsekwencji do obserwowanych zmian fenotypowych u mutanta *hvd14.d*. Dodatkowo, *MYB88* podlega regulacji przez BRI1 ETHYLMETHANE SULFONATE SUPPRESSOR1 (BES1), rozpoznany jako ko-regulator represorów SL u Arabidopsis (Liu et al., 2021).

W kolejnym kroku sekwencje promotorowe zidentyfikowanych DEG oraz genów kodujących DAP 2-tygodniowych i 4-tygodniowych siewek jęczmienia poddano analizie w celu identyfikacji motywów *cis*-regulatorowych oraz nad-reprezentowanych czynników transkrypcyjnych, które mogą wiązać się z tymi sekwencjami (Korek et al., 2025b). Podejście to ujawniło odpowiednio 70 i 75 czynników transkrypcyjnych potencjalnie zależnych od SL, które mogą regulować zmiany w transkryptomie i proteomie młodszych oraz starszych roślin. Zestawienie uzyskanych

danych pozwoliło na selekcję 33 czynników transkrypcyjnych wspólnych dla obu grup wiekowych. Adnotacja funkcjonalna wykazała, że czynniki te pełnią funkcje związane z regulacją hormonalną, co znajduje odzwierciedlenie w zaburzonej homeostazie hormonalnej obserwowanej u *hvd14.d*. Ponadto pięć z nich pokrywa się z czynnikami transkrypcyjnymi zależnymi od SL wytypowanymi w wyniku własnej analizy wcześniej opublikowanych danych dla Arabidopsis (Wang et al., 2020b), co sugeruje zachowaną konserwację mechanizmów regulacji zależnej od SL międzygatunkowo.

W obrębie grupy czynników transkrypcyjnych wspólnych dla jęczmienia i Arabidopsis szczególną uwagę zwraca TCP DOMAIN PROTEIN 21 (TCP21), będący integralnym elementem roślinnego zegara okołodobowego (Pruneda-Paz et al., 2009). TCP21, wspólnie z TIMING OF CAB EXPRESSION 1 (TOC1), tłumi transkrypcję *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) – głównego regulatora cyklu okołodobowego (Pruneda-Paz et al., 2009). Zegar okołodobowy wpływa na różnorodne procesy rozwojowe, w szczególności na kształtowanie architektury roślin (Gong et al., 2022). Ponadto, zarówno TCP21 jak i CCA1 zostały zidentyfikowane jako czynniki transkrypcyjne zależne od SL w osobnym badaniu, wyjaśniającym różnice w kontrastowym fenotypie mutantów *hvd14.d* oraz *hvd53a.f* (Korek et al., 2025a) (Rycina 7). Z kolei u ryżu wykazano, że OsCCA1 pozytywnie reguluje ekspresję *OsTB1*, *OsD14*, *OsD10* (uczestniczącego w biosyntezie SL), jednocześnie hamując rozwój zawiązków bocznych (Wang et al., 2020a). Ponadto, obniżenie lub zwiększenie ekspresji *OsCCA1* skutkuje odpowiednio zwiększeniem lub zmniejszeniem liczby źdźbeł, co odzwierciedla fenotypy obserwowane



Rycina 7. Proponowany mechanizm wyjaśniający różnice fenotypowe pomiędzy *hvd14.d* a *hvd53a.f*. CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) bezpośrednio reguluje transkrypcje genów *TEOSINE BRANCHED1* (*TB1*), *DWARF14* (*D14*) i *D10* poprzez wiązanie się z ich promotorami. Po rozpoznaniu cząsteczek strigolaktonów (SL) receptor D14 wchodzi w interakcję z białkiem F-box kompleksu SCF (SKP1-CULLIN-F-box), co prowadzi to do ubikwitynacji i następnie degradacji białka represorowego przez proteasom 26S. W konsekwencji geny *D10*, *D14* i *TB1* ulegają ekspresji hamując rozkrzewianie pędu.

u *hvd14.d* oraz *hvd53a.f*. Uzyskane wyniki, wraz z danymi literaturowymi, wskazują CCA1 jako silnego kandydata na czynnik transkrypcyjny zależny od SL, jednocześnie podkreślając istotną współzależność między sygnalizacją SL a zegarem okołodobowym.

Rola SL w regulacji tolerancji na suszę u jęczmienia

Poza dobrze udokumentowaną rolą w regulacji architektury pędu i systemu korzeniowego roślin, SL uczestniczą również w regulacji odpowiedzi na różnego rodzaju stresy abiotyczne (Alvi et al., 2022). Rośliny posiadające mutacje w genach kodujących białka zaangażowane w biosyntezę lub sygnalizację SL, w tym jęczmienny mutant *hvd14.d,* wykazują zwiększoną wrażliwość np. na niedobór wody (Marzec et al., 2020; Daszkowska-Golec et al., 2023). Szczególnie interesujące wydawało się zbadanie odpowiedzi na suszę u mutanta *hvd53a.f,* który wykazuje przeciwny fenotyp rozkrzewiania w porównaniu do *hvd14.d* (Korek et al., 2025a). Ponieważ *hvd53a.f* już w warunkach kontrolnych wykazuje obniżoną zawartość chlorofilu oraz ograniczoną wydajność fotosyntezy, analizę skupiono na ocenie zmian zachodzących w fotosyntezie pod wpływem stresu wodnego. Stres suszy znacząco wpływa na reakcje fotosyntezy zależne od światła, które zachodzą w błonach tylakoidów, gdzie chlorofil wychwytuje energię świetlną, inicjując produkcję ATP i NADPH za pośrednictwem odpowiednio fotosystemu II i fotosystemu I (Chauhan et al., 2023). Biorąc pod uwagę wcześniejsze obserwacje, podjęto próbę odpowiedzi na pytanie, czy stres suszy dodatkowo pogłębia te niekorzystne zjawiska u roślin *hvd53a.f*.

Ze względu na zwiększoną wrażliwość *hvd14.d* na stres suszy, rośliny tego mutanta włączono do analiz jako dodatkowy punkt odniesienia. Analizowane genotypy - *hvd53a.f, hvd14.d* oraz WT - uprawiano przez 10 dni przy optymalnej wilgotności gleby (14% vwc), po czym przez kolejne 5 dni wstrzymano podlewanie, aż wilgotność spadła do 3%. Następnie przez 10 dni stosowano silny stres suszy (1,5–3% vwc). Rośliny kontrolne uprawiano równolegle w tych samych warunkach, utrzymując optymalną wilgotność gleby (14% vwc). Analiza obejmująca szereg parametrów fizjologicznych - w tym suchą masę, względną zawartość wody (RWC), poziom chlorofilu, wskaźnik wydajności fotosyntezy (PIabs), rozproszenie energii (DI/RC), liczbę centrów reakcji (RC/CS) oraz barwienie DAB - wykazała, że linia *hvd53a.f* cechuje się mniejszą wrażliwością na niedobór wody w porównaniu do WT, jak i nadwrażliwego na suszę *hvd14.d* (Tabela 1) (Korek et al., 2025a). Warto jednak zaznaczyć, że bezwzględne

wartości parametrów opisujących wydajność fotosyntezy w przypadku *hvd53a.f* były najniższe spośród wszystkich badanych genotypów w warunkach kontrolnych oraz podczas suszy. Wyniki te sugerują, że rośliny *hvd53a.f* wykazują większą tolerancję na stres suszy kosztem obniżenia wydajności fotosyntezy, którą jednak utrzymują na stabilnie niskim poziomie. Zwiększoną tolerancję na stres suszy odnotowano również w przypadku potrójnego mutanta *smxl6,7,8* u Arabidopsis, gdzie była ona powiązana m.in. z wyższą zdolnością do detoksykacji ROS oraz z odkładaniem grubszych warstw kutykuli, ograniczających utratę wody (Li et al., 2020b).

Tabela 1. Zmiany parametrów fizjologicznych w odpowiedzi na suszę u mutantów jęczmienia *hvd14.d* i *hvd53a.f* oraz WT. Zmiany wyliczono jako procent [%] względem kontroli.

Genotyp	Warunki	Sucha masa [mg]	RWC [%]	Zawartość chlorofilu [a. u]	PIabs [a. u]	DI/RC* [a. u]	RC/CS [a. u]
hvd14.d	kontrola	371,65	83,16	42,04	4,63	0,33	870,94
	susza	48,92	38,5	28,06	2,67	0,53	637,74
	%	13,16	46,30	66,75	57,67	160,61	73,22
WT	kontrola	437,01	81,4	42,17	4,49	0,32	916,09
	susza	141,67	58,83	33,98	3,82	0,39	828,38
	%	32,42	72,27	80,58	85,08	121,88	90,43
hvd53a.f	kontrola	134,8	81,65	22,19	0,88	1,89	328,97
	susza	95,24	71,95	19,83	0,47	1,7	310,19
	%	70,65	88,12	89,36	53,41	89,95	94,29

*Wyższe wartości wskaźnika DI/RC świadczą o większych stratach energii w postaci ciepła, a. u – jednostki arbitralne

Aby lepiej zrozumieć molekularne podstawy tej zróżnicowanej odpowiedzi na stres suszy, przeprowadzono analizę transkryptomiczną badanych genotypów. Mutant *hvd53a.f* narażony na stres suszy wykazał najmniejszą liczbę DEG (5043) w porównaniu do warunków kontrolnych, co sugeruje bardziej efektywną lub już wstępnie przygotowaną odpowiedź na niedobór wody. Przeciwnie, u *hvd14.d* zaobserwowano największą liczbę DEG (9099), co może odzwierciedlać intensywną konieczność re-programowania transkryptomu w warunkach stresowych, wynikającą z jego wyższej wrażliwości na suszę. Wśród 137 genów o przeciwstawnym wzorcu ekspresji – podwyższonej u *hvd53a.f* i obniżonej u *hvd14.d* – zidentyfikowano gen

HORVU.MOREX.r2.6HG0458250 kodujący akwaporynę błony plazmatycznej Plasma Membrane Intrinsic Protein 2-5 (PIP2-5), potencjalnie odgrywającą istotną rolę w różnicach dotyczących gospodarki wodnej pomiędzy analizowanymi mutantami. Wykazano, że rośliny Arabidopsis z nadekpresją genu *HvPIP2-5* były zdolne do przetrwania i regeneracji po 3-tygodniowym okresie suszy, w przeciwieństwie do roślin kontrolnych (Alavilli et al., 2016). Nadekspresja *HvPIP2-5* sprzyjała również utrzymaniu stabilnego poziomu chlorofilu, retencji wody oraz niższemu nagromadzeniu ROS w warunkach stresu solnego i osmotycznego. Analogiczne obserwacje odnotowano u mutanta jęczmienia *hvd53a.f*, co sugeruje, że zwiększona, zależna od SL, ekspresja *HvPIP2-5* genu może przyczyniać się do poprawy tolerancji na stresy abiotyczne poprzez regulację gospodarki wodnej i stresu oksydacyjnego.

W dalszej kolejności przeprowadzono bioinformatyczną identyfikację czynników transkrypcyjnych powiązanych z sygnalizacją SL, które mogłyby wyjaśniać kontrastujące fenotypy *hvd14.d* i *hvd53a.f* w warunkach stresu suszy. Analiza pozwoliła wyodrębnić grupę czynników transkrypcyjnych regulujących ekspresję DEG specyficznych dla *hvd14.d* lub *hvd53a.f*, spośród których dwa okazały się wspólne i zaangażowane w regulację tolerancji na stresy abiotyczne (Korek et al., 2025a). Pierwszy z nich, JUNGBRUNNEN 1 (JUB1) kontroluje ekspresję szeregu genów odpowiedzialnych za reakcję na obecność ROS, w tym genów kodujących białka szoku cieplnego i transferazy S-glutationowe, które są kluczowe dla utrzymania równowagi redoks w komórkach i odporności na stres (Wu et al., 2012). W efekcie, zmniejszane są uszkodzenia oksydacyjne w warunkach suszy, co sprzyja przeżywalności i adaptacji roślin. Barwienie DAB, potwierdziło znacznie silniejszą zdolność mutanta *hvd53a.f* do detoksykacji ROS, co może stanowić kluczowy element mechanizmu jego zwiększonej tolerancji na stres suszy, wynikający z bardziej efektywnej aktywacji szlaków antyoksydacyjnych regulowanych przez JUB1. Ponadto JUB1 wiąże się z promotorem i reguluje ekspresję *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A* (*DREB2A*), genu kodującego kluczowy czynnik transkrypcyjny zaangażowanego w retencję wody w komórkach w warunkach stresu suszy (Ebrahimian-Motlagh et al., 2017). Drugi zidentyfikowany czynnik transkrypcyjny, DREB2H, należy do tej samej rodziny białek, sugerując możliwość współdziałania obu regulatorów w zwiększaniu tolerancji roślin na niedobór wody.

Wyniki te wskazują, że zarówno mechanizmy detoksykacji ROS, jak i regulacja gospodarki wodnej mogą stanowić istotny element adaptacji zależnej od SL, która zakłócona u *hvd14.d* prowadzi do zwiększonej wrażliwości na suszę, natomiast jej aktywacja u *hvd53a.f* może przyczyniać się do zwiększonej tolerancji.

Podsumowanie

W ramach przeprowadzonych analiz zidentyfikowano znaczną liczbę potencjalnych czynników transkrypcyjnych zależnych od SL, które mogą regulować wzrost i rozwój siewek jęczmienia, a także uczestniczyć w adaptacji roślin do stresu suszy. Na podstawie adnotacji funkcjonalnej oraz dostępnych danych literaturowych wykazano, że zidentyfikowane czynniki transkrypcyjne biorą udział m.in. w odpowiedzi na hormony, biosyntezie kutyny, różnicowaniu aparatów szparkowych, regulacji zegara okołodobowego, homeostazie ROS oraz retencji wody. Uzyskane wyniki wskazują również na zachowaną konserwację mechanizmów regulacji zależnych od SL między gatunkami jednoliściennymi oraz dwuliściennymi, co może stanowić istotny punkt wyjścia do dalszych analiz. W perspektywie kontynuacji prowadzonych badań niezbędne jest przeprowadzenie funkcjonalnej weryfikacji wytypowanych czynników transkrypcyjnych, aby określić ich dokładną rolę w szlakach sygnalizacyjnych SL. Uzyskane w niniejszej rozprawie doktorskiej wyniki pogłębiają rozumienie roli SL u jęczmienia i stanowią podstawę do dalszych badań nad rolą tych hormonów oraz ich potencjałem w modyfikowaniu cech użytkowych roślin.

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ROZDZIAŁ II

Wnioski

Na podstawie wyników badań przedstawionych w rozprawie doktorskiej można zaproponować następujące wnioski:

1. Mutacja w genie *HvD14*, kodującym receptor SL u jęczmienia, prowadzi do niewrażliwości na SL, a przez to do większego rozkrzewienia części nadziemnej, niższej wysokości roślin oraz większej wrażliwości na stres suszy, w porównaniu do roślin typu dzikiego.

2. Mutacja w genie *HvD53A* kodującym represor SL u jęczmienia prowadzi do ciągłej aktywności genów zależnych od SL, a przez to zmniejszenia rozkrzewienia części nadziemnej, zwiększonej wysokości roślin oraz zwiększenia tolerancji na stres suszy w porównaniu do roślin typu dzikiego.

3. Niewrażliwość na SL, spowodowana mutacją w receptorze D14, skutkuje zaburzeniem hormonalnej homeostazy w pędzie rozwijających się roślin, co znajduje wyraz w zmienionych profilach najważniejszych klas hormonów, a także innymi wzorami ekspresji genów oraz obecności białek związanych z biosyntezą bądź sygnalizacją tych fitohormonów, w porównaniu do roślin typu dzikiego. Zmiany te są dynamiczne w trakcie rozwoju roślin jęczmienia.

5. Szlak sygnalizacji SL reguluje ekspresję genów specyficznych oraz wspólnych dla pędu i korzenia siewek jęczmienia, co sugeruje istnienie zarówno organo-specyficznych, jak i współdzielonych mechanizmów regulacyjnych zależnych od SL odpowiadających za rozwój rośliny.

5. CIRCADIAN CLOCK ASSICIATED 1 może pełnić funkcję czynnika transkrypcyjnego zależnego od SL, łącząc szlak sygnalizacji SL z cyklem okołodobowym, co może tłumaczyć różnice w tempie dojrzewania i kwitnienia pomiędzy mutantami *hvd14.d* i *hvd53a.f* a roślinami typu dzikiego.

4. Czynniki transkrypcyjne, takie jak, BASIC PENTACYSTEINE 6 (BPC6), TCP DOMAIN PROTEIN 21 (TCP21), HIGH CAMBIAL ACTIVITY 2 (HCA2), BES1-INTERACTING MYC-LIKE 2 (BIM2) i PISTILLATA (PI) mogą pośredniczyć w transdukcji sygnału SL zarówno u roślin jednoliściennych (jęczmień), jak i dwuliściennych (rzodkiewnik pospolity).

7. Rośliny *hvd53a.f* wykazują większą tolerancję na stres suszy kosztem obniżenia wydajności fotosyntezy, którą jednak utrzymują na stabilnie niskim poziomie. Kontrastujące fenotypy *hvd14.d* i *hvd53a.f* w warunkach stresu suszy, może wyjaśniać aktywność transkrypcyjna JUNGBRUNNEN 1 potencjalnie zależnego od SL.

8. Wytypowane w ramach rozprawy doktorskiej czynniki transkrypcyjne potencjalnie zaangażowane w szlak sygnalizacji SL stanowią wartościową bazę do dalszych badań funkcjonalnych, umożliwiających pełniejsze poznanie mechanizmów molekularnych regulujących rozwój oraz adaptację jęczmienia do zmiennych warunków środowiskowych.

ROZDZIAŁ III

Publikacje wchodzące w skład rozprawy

ROZDZIAŁ III.1

Strigolactones and abscisic acid interactions affect plant development and response to abiotic stresses.

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Strigolactones and abscisic acid interactions affect plant development and response to abiotic stresses

Magdalena Korek[1*] and Marek Marzec[1]

Abstract

Strigolactones (SL) are the youngest group of plant hormones responsible for shaping plant architecture, especially the branching of shoots. However, recent studies provided new insights into the functioning of SL, confirming their participation in regulating the plant response to various types of abiotic stresses, including water deficit, soil salinity and osmotic stress. On the other hand, abscisic acid (ABA), commonly referred as a stress hormone, is the molecule that crucially controls the plant response to adverse environmental conditions. Since the SL and ABA share a common precursor in their biosynthetic pathways, the interaction between both phytohormones has been largely studied in the literature. Under optimal growth conditions, the balance between ABA and SL content is maintained to ensure proper plant development. At the same time, the water deficit tends to inhibit SL accumulation in the roots, which serves as a sensing mechanism for drought, and empowers the ABA production, which is necessary for plant defense responses. The SL-ABA cross-talk at the signaling level, especially regarding the closing of the stomata under drought conditions, still remains poorly understood. Enhanced SL content in shoots is likely to stimulate the plant sensitivity to ABA, thus reducing the stomatal conductance and improving the plant survival rate. Besides, it was proposed that SL might promote the closing of stomata in an ABA-independent way. Here, we summarize the current knowledge regarding the SL and ABA interactions by providing new insights into the function, perception and regulation of both phytohormones during abiotic stress response of plants, as well as revealing the gaps in the current knowledge of SL-ABA cross-talk.

Keywords Abiotic stress, Abscisic acid, Phytohormone cross-talk, Plant development, Strigolactones

Background

Phytohormones (plant hormones) are a group of naturally occurring, organic chemical compounds produced by plants in micromolar concentrations however, they significantly affect the entire life cycle of plants, from early embryogenesis to senescence [1]. Plant hormones act as chemical messengers coordinating the molecular pathways that lead to the growth and development of the organisms. Several members of the phytohormone family have already been identified, including abscisic acid (ABA), auxins (AUX), brassinosteroids (BR), cytokinins (CKs), ethylene (ET), gibberellins (GA), jasmonates (JA), and strigolactones (SL) [2]. Due to the sessile lifestyle, plants are constantly subjected to a wide range of biotic and abiotic stresses [3]. To adapt to such adverse situations, plants developed various mechanisms that allow them to perceive the stress stimulus and consequently to provide adequate defense reactions. When faced with

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unfavourable environmental conditions, plants require the activation of a complex signaling network, where phytohormones play a critical role [4]. Interestingly, individual hormones can interact with each other to ensure plant stress tolerance. These interactions can occur at the hormone biosynthesis or signaling level and could be both stimulatory and inhibitory in nature [5–7]. Here, we present a comprehensive overview of the cross-talk between ABA, commonly referred to as the stress hormone, and SL, the youngest member of phytohormone family, which is increasingly confirmed to play a role in the plant's response to abiotic stresses.

SL: a brief overview

Initially identified as rhizosphere signaling molecules, SL were first identified from cotton (*Gossypium arboreum*) root exudate in the 1960s and were found to induce germination of parasitic seeds such as the witchweeds (*Striga spp.*) and broomrapes (*Orobanche* and *Phelipanche spp*) [8]. For this reason, the recognized molecule was named strigol. Later, it was shown that SL exuded by plant roots trigger hyphae branching of mycorrhizal fungi, thus increasing the chances of contact between symbionts [9]. More recent studies provided a better understanding of SL function as a direct regulator of plant growth. In 2008, the inclusion of SL in the list of plant hormones was supported by the analysis of mutants that exhibited semi-dwarf and highly shoot branching phenotypes in three genetically distant model plant species, such as arabidopsis (*Arabidopsis thaliana*), pea (*Pisum sativum*), and rice (*Oryza sativa*) [10, 11]. The studies confirmed that treatment with a synthetic analogous of SL rescued the phenotype of SL-depleted plants, which was not possible with SL-insensitive mutants. Further, the impact of SL on shaping the above-ground plant architecture was also proved in other species [12, 13]. Up to now, semi-dwarf and highly branched mutants affected in SL-biosynthesis or signaling pathway have been identified from a wide range of species, including arabidopsis (*more axillary growth, max*) [14–17], petunia (*Petunia hybrid*; d*ecreased apical dominance, dad*) [18–22], pea (*Pisum sativum; ramousus, rms*) [23, 24] and rice (*high-tillering dwarf, htd; dwarf, d*) [25, 26].

SL are primarily synthesized in the roots and subsequently transported to the above-ground parts of the plant [27]. The initial step in SL biosynthesis is the conversion of all-*trans*-β-carotene to carlactone (Fig. 1). This process is carried out in plastids and involves three enzyme players - carotenoid isomerase (D27) and two carotenoid cleavage dioxygenases (CAROTENOID CLEAVAGE DIOXYGENASE7/8; CCD7, CCD8) [28]. Another step occurs in the cytoplasm and is led by MAX1-type monooxygenase, transforming carlactone into carlactonoic acid (CLA), giving rise to other SL and SL-like compounds. The subsequent steps of SL biosynthesis vary across plant species [29]. In arabidopsis, maize (*Zea mays*) and tomato (*Solanum lycopersicum*) research, it was revealed that carlactonoic acid is further transformed by CLA methyltransferase (CLAMT) to methyl carlactonoate (MeCLA), which is the key intermediate for non-canonical SL [30]. On the other hand, enzymes from the CYP722C subfamily have been shown to form canonical SL in cowpea (*Vigna unguiculate*), tomato, cotton, and Lotus japonicus [31]. Canonical SL have a tricyclic lactone structure composed of three rings (ABC-rings) connected to a butenolide group (D-ring) via an enol-ether bridge [32]. Rings A and B differ due to the additional functional groups (i.e. $-CH_3$, –OH, $-C(O)CH_3$), while rings C and D are highly conserved and play an essential role in the biological activity of SL molecules [33]. Canonical SL are further divided into strigol- and orobanchol-type classes based on the stereochemistry of C-ring, which may be a β- and an α-oriented, respectively [34]. At the same time, both subgroups share the 2'R orientation [35]. In the research area, the most commonly used synthetic analogue of SL is *rac*-GR24. This compound is an equimolar mixture of the two enantiomers: $GR24^{5DS}$ that mimics the configuration and activity of the natural 5-deoxystrigol (5DS) and $GR24^{ent-5DS}$ with stereochemistry at 2'*S* not occurring in natural SL [27]. During the chemical synthesis of GR24, the two orobanchol-type enantiomers are also produced however, these compounds are not usually involved in biological assay [36]. It is crucial that $GR24^{ent-5DS}$ is also perceived by KARRIKIN INSENSITIVE 2 (KAI2), a receptor involved in karrikin (KAR) signaling. Thus the results obtained with the usage of *rac*-GR24 might be ambiguous due to the stimulation of both SL and KAR pathways [36]. To activate the SL transduction exclusively, the use $GR24^{5DS}$ or recently synthetized $GR24^{4DO}$ is recomended [37]. In contrast to canonical SL, non-canonical SL are very diverse in the structure of their ABC-rings, but possess both an enol-ether bridge and D-ring moieties. Studies have demonstrated that a single plant species can generate various types of SL [38]. Furthermore, it has been suggested that SL can result in different physiological responses in plants depending on their chemical composition [39–41]. The fact that canonical SL are found only in limited plant species, and their specific and stereoselective movement from roots to shoots, indicates that the plant hormones responsible for suppressing shoot branching might be non-canonical SL, and not canonical SL [41–43]. To date, more than 30 naturally occurring SL have been identified among mono- and dicotyledonous plants serving many roles in plant growth and development [29]. Experimental studies have confirmed the involvement of SL in a range of processes such as parasitic seed germination, early seedling

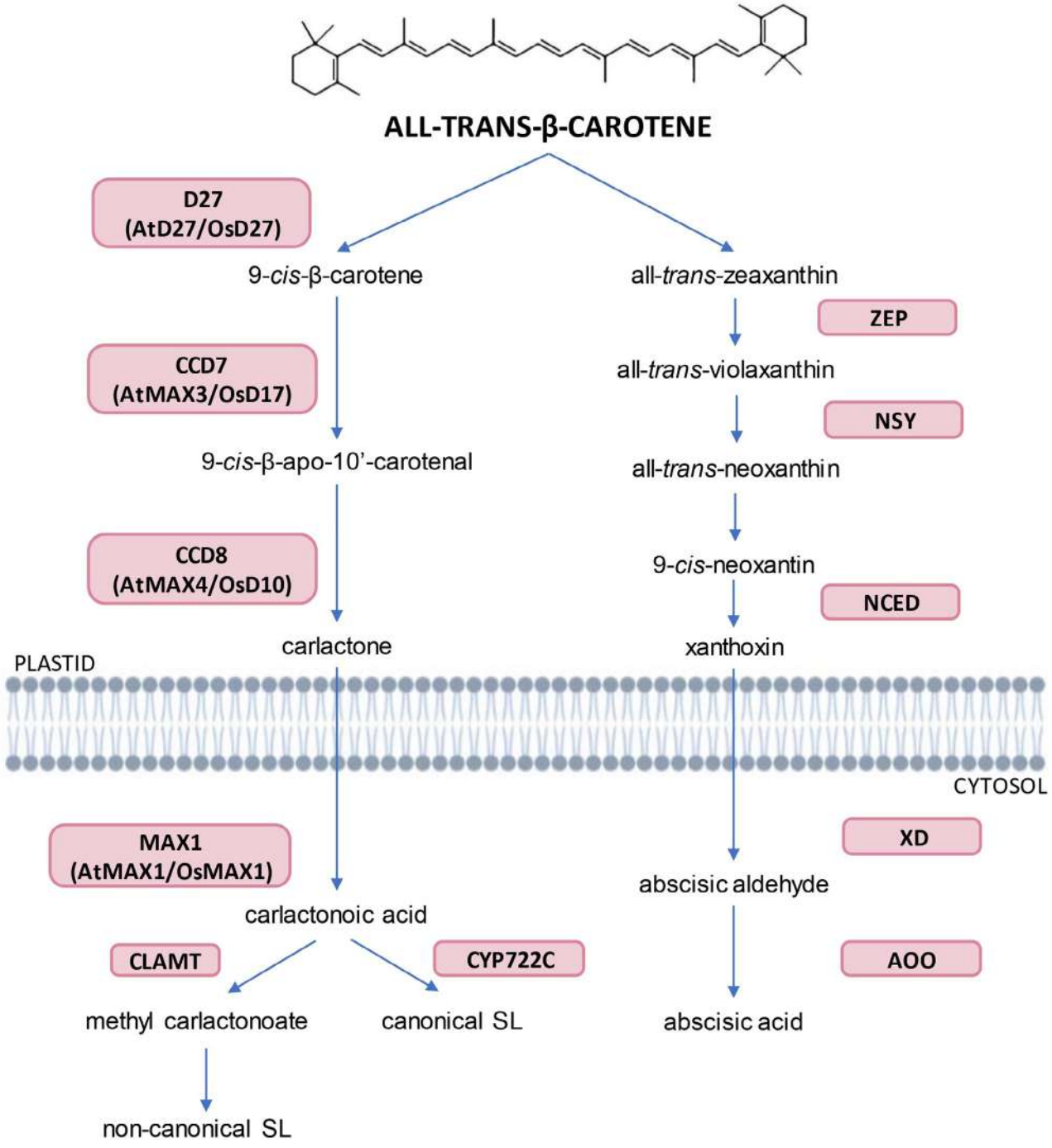

Fig. 1 The biosynthetic pathways of strigolactones (SL) and abscisic acid (ABA) share a common precursor. The formation of SL starts with the isomerization of all-*trans*-β-carotene by the DWARF 27 (D27) at the C-9 position. Next, two CAROTENOID CLEAVAGE DIOXYGENASEs – CCD7 and CDD8 convert 9-*cis*-β-carotene to carlactone, which is further oxidized by cytochrome P450 monooxygenases, such as MORE AXILLARY GROWTH 1 (MAX1). The carlactonoic acid (CLA) undergoes further reactions either by CLA methyltransferase (CLAMT) to form a methyl carlactonoate, which is a key intermediate for non-canonical SL, or by enzymes from CYP722C subfamily producing canonical SL. The ABA biosynthesis part that takes place in the plastid requires a series of enzymatic reactions that lead to the formation of xanthoxin. Then xanthoxin is transported to cytosol, converted to abscisic aldehyde by XANTHOXIN DEHYDROGENASE (XD), and further oxidized by ABSCISIC ALDEHYDE OXIDASE (AAO) to ABA. Created with BioRender.com

development, leaf senescence and control of main and lateral root or root-hair elongation [44, 45]. Besides these developmental processes, there is a growing body of evidence suggesting that SL also participate in the plant's response to various biotic and abiotic stresses. Specifically, the activity of SL has been documented during the plant's response to suboptimal environmental conditions such as drought, salinity, high or low temperature, nutrient deficiency, oxidative stress, and fluctuations in light quality and intensity [46, 47]. Moreover, there have been postulations about the potential role of SL in plant's defense to pathogens [48]. Recent reports have shed light on the molecular mechanisms underlying the involvement of SL in stress responses, highlighting their potential as targets for improving plant tolerance to environmental stressors [31, 49].

In the last decade, various breakthroughs have been made in scientific research regarding the perception and signaling of the SL. All major SL signal transduction pathways components were already described in arabidopsis and rice [50]. Similar to most phytohormones, the mechanism for transducing the SL signal is based on the degradation of repressor protein (Fig. 2A). The first step of the cascade perception is recognizing and binding the SL molecules by the receptor (AtD14/OsD14), which belongs to the α/β hydrolase protein family [51] (Fig. 2B). This interaction results in conformation changes of the D14, which is necessary for the interaction between receptor and other components from SL signaling complex [52]. The receptor with altered conformation can bind the F-box protein (AtMAX2/OsD3) from the SKP1-CULLIN-F-BOX complex (SCF) and the SL repressor (SUPPRESSORS OF MAX2 1-LIKE6, 7, 8, AtSMXL6,7,8/OsD53) [53]. Following, the degradation of the SL repressor results in the activation of transcription factors (TFs) related to SL [54]. Recently, *Arabidopsis* transcriptomic studies revealed that exogenous SL may activate 24 genes and repress 14 genes encoding TFs, respectively. The effect of SL-dependent responsiveness was experimentally confirmed in three of them – *BRANCHED1* (*BRC1*), *TCP DOMAIN PROTEIN1* (*TCP1*) and *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*), whose roles are related to the control of shoot branching, leaf shape, and anthocyanin biosynthesis [55]. Interestingly, it was also shown that SMXL6 targeted promoter regions of *SMXL6,7,8*, indicating that this SL repressor protein functions as a self-regulating TF, which may also control the expression of other *SMXL* genes.

ABA: a brief overview

Abscisic acid (ABA) was discovered in the early 1960s by two independent research groups from the United States and the United Kingdom. While Eagles et al. identified a molecule that can trigger dormancy and called it dormin [56], Ohkuma et al. isolated an abscission-accelerating factor from cotton fruits, which they called abscisin II [57]. Both discovered chemical compounds turned out to have the same chemical structure [58]. Therefore, the newly-recognized molecule was renamed abscisic acid to standardise the nomenclature. In contrast to SL, the structure of ABA is conserved through plant kingdom [35]. From a chemical point of view, ABA is a 15-carbon molecule classified as a sesquiterpenoid formed by joining three isoprenoid units [59]. The *trans-* or *cis-* stereoisomerization is determined by the orientation of the carboxyl moiety at position 2'. Moreover, the presence of an asymmetric carbon atom 1' decides about the S(+) or R(-) enantiomers [60]. Naturally occurring ABA is mainly found in plants as (S)-*cis*-ABA [61]. ABA is mostly synthesized in mature leaves (phloem companion cells, guard cells, and mesophyll cells), but also in roots, flowers, fruits, and seeds [62]. Due to specific phenotype such as precocious germination of seeds and wilted appearance of the plants, mutants insufficient in ABA biosynthesis were isolated from numerous plant species, including arabidopsis, barley (*Hordeum vulgare*), tomato, tobacco (*Nicotiana tabacum*) and maize [63]. ABA, similarly to SL, is a derivative of all-*trans*-β-carotene, thus the first steps of enzymatic reactions take place in plastids (Fig. 1). The process starts with the hydroxylation of all-*trans*-β-carotene to all-*trans*-zeaxanthin, which is later converted to all-*trans*-violaxanthin by ZEAXANTIN EPOXIDASE (ZEP) [64]. Following, NEOXANTIN SYNTHETASE (NSY) transforms all-*trans*-violaxanthin to all-*trans*-neoxanthin, then isomerized to 9-*cis*-neoxantin [65]. The last step of the biosynthetic pathway that occurs in the plastids is led by EPOXYCAROTENOID DIOXYGENASE (NCED) and results in cleavage of 9-*cis*-neoxanthin to xanthoxin (Fig. 1). This is the only non-reversible reaction and is believed to be a key rate-limiting point in the biosynthesis process [66]. Further, xanthoxin is transported to the cytosol, where it is converted to abscisic aldehyde by XANTHOXIN DEHYDROGENASE (XD). The final step is led by ABSCISIC ALDEHYDE OXIDASE (AAO) and results in oxidation of abscisic aldehyde to ABA (Fig. 1) [67].

It has become progressively clear that ABA plays a dual role in the plants' life cycle as a plant growth regulator and an improving stress tolerance factor depending on the relative endogenous concentrations of ABA [62]. Under optimal environmental conditions, it has been demonstrated that low concentrations of ABA regulate plants' vegetative growth, including seed development and germination, embryo maturation, root architecture, bud dormancy, fruit ripening, and leaf abscission [68]. Conversely, enhanced amounts of ABA play an essential role in plants' adaptation to a varied range of stresses such as heat or cold stress, high level of solid salinity,

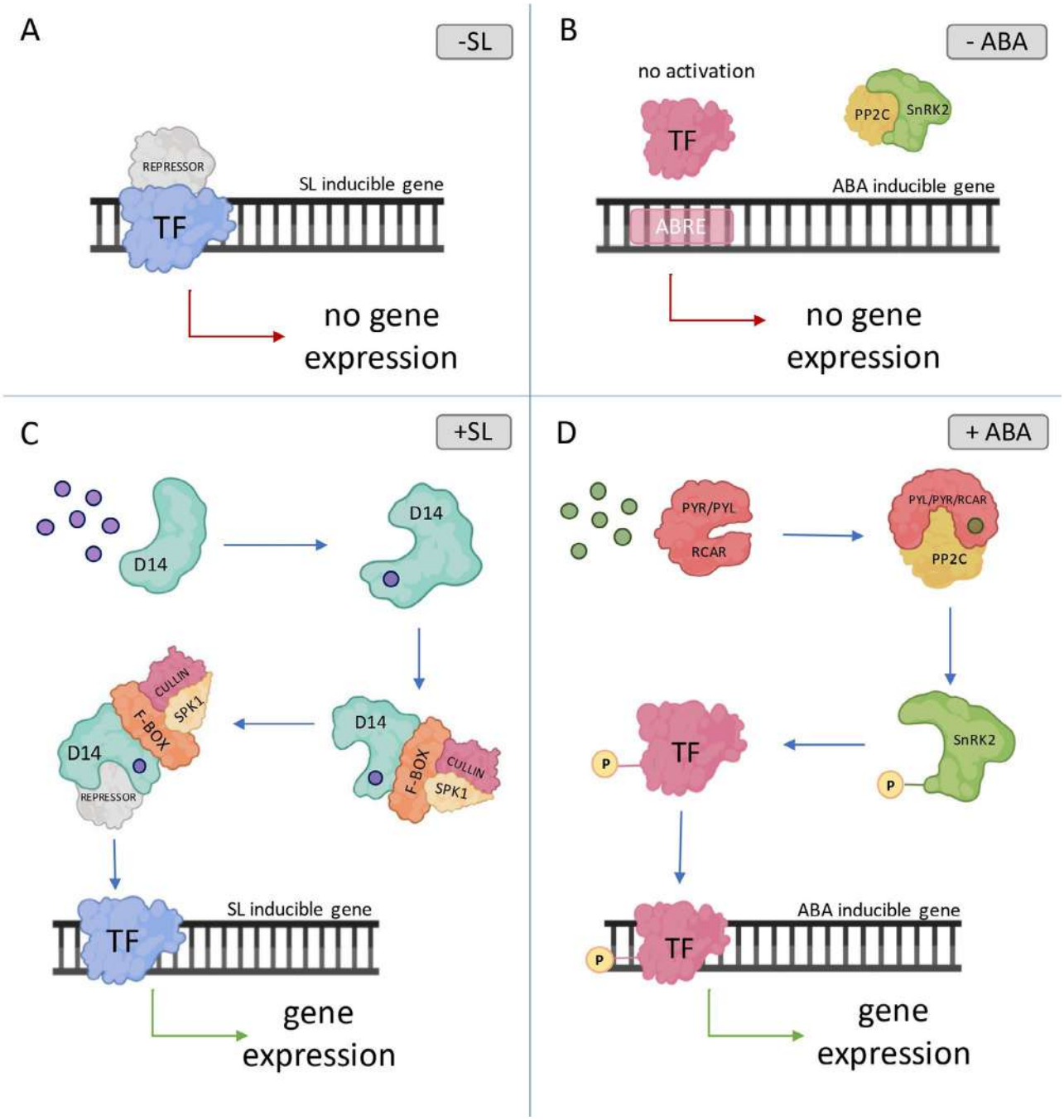

Fig. 2 Perception and signaling of strigolactones (SL) and abscisic acid (ABA). **A**) In the absence of SL, the expression of SL inducible gene is blocked by repressor. **C**) The SL molecules are recognized and bound by D14 protein, which results in conformational changes of SL receptor. Following, the D14 protein interacts with the F-box protein from the SCF complex and the SL repressor, resulting in degradation of SL repressor. As a consequence, the transcription of SL inducible gene is activated. **B**) In the absence of ABA, the TF remains inactive as the interaction between PP2C and SnRK2 blocks its phosphorylation. **D**) When ABA molecules are recognized and bound by ABA receptor (PYL/PYR/RCAR), the receptor undergoes a conformational change. This change enables the ABA receptor to interact with the PP2C protein, which then releases the SnRK2. The SnRK2 is subsequently autophosphorylated or phosphorylated by other proteins, resulting in the activation of TF. Once activated, the TF can bind to ABRE elements in the promoter of ABA inducible gene and recruit transcriptional machinery. TF – transcription factor, PP2C - PROTEIN PHOSPHATASE 2 C, PYR - PYRABACITN RESISTANCE, PYL - PYRABACTIN RESISTANCE 1-LIKE, RCAR - REGULATORY COMPONENT OF ABA RECEPTOR, SnRK2 - SUCROSE NONFERMENTING 1 RELATED PROTEIN KINASES 2, ABRE – ABA responsive element, D14 – DWARF 14, SCF – SPK1-CULLIN-F-BOX, P – phosphorus residue. Created with BioRender.com

and abundant heavy metals [69]. One of the most well-known and fundamental actions of ABA is to control the stomatal closure during drought stress, which is critical for maintaining water retention in the plant [70]. As the main phytohormone acting against abiotic stresses, the fluctuation of endogenous ABA levels must be consistently triggered by the balance between biosynthesis and catabolism due to changing environmental conditions [71]. ABA catabolism is generally categorized into two types of reactions, conjugation and hydroxylation [72]. The most widespread form of conjugated ABA is ABA-glucosyl ester (ABA-GE), which is biologically inactive. However, recent studies indicate that ABA-GE may act as a reservoir of active ABA in dehydration conditions through one-step hydrolysis by β-glucosidase [73]. The predominant and non-reversible enzymatic reaction leading to ABA catabolism is 8'-hydroxylation led by CYP707As, cytochrome P450 monooxygenases.

The pathway for ABA signal transduction requires three main classes of proteins; ABA receptors named PYRABACITN RESISTANCE/PYRABACTIN RESISTANCE 1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR), ABA repressors from the PROTEIN PHOSPHATASE 2C (PP2Cs) group A family, and the SUCROSE NONFERMENTING 1 RELATED PROTEIN KINASES 2 (SnRK2s) as a positive regulators [74]. When ABA is absent, a physical association exists between PP2Cs and SnRK2s. This interaction has an inhibitory effect on the phosphorylation activity of SnRK2s. Consequently, ABA signal transduction is blocked, preventing the activation of downstream TFs [59] (Fig. 2C). In the case of ABA presence, the hormone is perceived and bound by PYR/PYL/RCAR receptors, which changes the receptor's conformation and allows for the interaction between receptor and PP2Cs catalytic site. This interaction suppresses the phosphatase activity of ABA repressor proteins and relieves the inhibition of SnRK2s [75]. The released SnRK2s are then activated by autophosphorylation or phosphorylation by other proteins, and further SnRK2s are able to phosphorylate downstream proteins or TFs that induce ABA responses [76] (Fig. 2D). The activated ABA-related TFs directly bind to ABA-responsive element (ABRE) – (ACGTGG/TC), a major *cis*-element in the promoters of ABA-responsive genes [77]. The phosphorylation/dephosphorylation is a key process controlling ABA signal transduction and activation of ABA-responsive genes. In addition, ubiquitination and degradation of core proteins in ABA signaling pathway by the ubiquitin proteasome system (UPS) is also a critical step that modulates the signal relay [78]. Protein degradation by the UPS is a regulatory mechanism studied during various aspects of ABA stress response. So far, over 20 proteins with E3 ligase activity have been identified that regulate the protein level of ABA signaling core components, including ABA receptors, PP2Cs proteins and ABA-responsive TFs [79].

Interactions between SL and ABA biosynthesis pathways during plant growth and development

All-*trans*-β-carotene is a molecule that undergoes a cascade of enzymatic reactions leading to the formation of both SL and ABA phytohormones (Fig. 1). The *TILLERING 20* (*T20*) gene, which encodes an isomerase involved in carotenoid biosynthesis has been functionally analyzed to prove that SL and ABA share a common precursor. Loss-of-function mutation in the *T20* gene reduced both SL and ABA levels in rice plants [80]. Therefore, it raises the question of whether SL and ABA interact with each other at the biosynthetic level to maintain hormone homeostasis.

In 2015 an *in silico* analysis showed that *cis*-regulatory elements in promoters of arabidopsis and rice SL biosynthesis genes are related to hormonal regulation [81]. Most of them are connected with ABA-responsive factors, which clearly emphasizes that the biosynthesis of SL may be ABA-dependent. Indeed, several reports on various plant species suggest the role of ABA in regulating SL biosynthesis. The spatial-temporal expression pattern of a reporter gene controlled by the native AtD27 promoter (*pAtD27:NLS-GUS*) enhanced in primary and lateral roots of 7-day-old arabidopsis seedlings after ABA treatment. RT-qPCR further confirmed this observation, showing an increase in *AtD27* expression caused by ABA application [82] (Supplementary Table 1). In another research, a noteworthy increase in the relative transcripts levels of arabidopsis *CCD7* and *CCD8* SL-biosynthesis genes in leaves was observed 1 h after ABA treatment, with the maximum level of increased expression of both genes reached after 10 hours [83]. Similar correlations were observed for tomato seedlings, where treatment with NCED inhibitor abamineSG reduced ABA and SL content in roots compared to non-treated plants [84]. Comparable results were also found in tomato ABA-deficient mutants, such as *notabilis* (mutation in *NCED* gene), *sitiens* and *flacca* (mutations in *AAO* enzyme). The endogenous content of both SL and ABA was much lower in analyzed mutants than in wild-type (WT) plants [84]. In contrast, applying the carotenoid cleavage dioxygenase inhibitor D2 reduced SL but not ABA content in roots [84]. The effect of limiting SL biosynthesis due to inhibited ABA production was also noted in monocotyledonous plants. The root exudates of maize plants with a null mutation in the *ZmNCED1* gene contributed to a significant reduction in the germination of parasitic seeds, and this outcome is suggested to be a result of low SL content [85]. All this together clearly highlights the positive impact of ABA on SL biosynthesis under optimal plant growth conditions. Notably, a stimulating effect

of SL on the activity of ABA biosynthesis genes was also demonstrated. In rice, five NCED genes are believed to be involved in ABA biosynthesis [86]. After treating rice seedlings with *rac*-GR24, the expression of *OsNCED1* and *OsNCED2* was significantly induced in shoot bases, while the activity of *OsNCED3* was enhanced in roots. However, the expression level of *OsNCED4 and OsNCED5* remained unchanged [80] (Supplementary Table 1). These results suggest that different *NCED* genes might be involved in ABA biosynthesis in an organ-specific manner, and some may be SL-activated.

Since SL and ABA share a common precursor, it was initially assumed that their relationship should be competitive rather than promoting. However, recent research showed that D27 might also stimulate ABA biosynthesis. The shoot ABA content was significantly increased in two independent rice lines overexpressing the *OsD27* gene compared with WT. Furthermore, it was observed that mutation in the *OsD27* gene resulted in untouched ABA levels in rice shoots, in contrast to other SL-deficient mutants, where ABA accumulation was increased [87]. Interestingly, the induced expression of the *OsD27* gene was demonstrated in both *Osccd7* and *Osccd8* mutants. If *D27* actually promotes ABA amounts, then the enhanced levels of *D27* transcripts followed by increased levels of ABA in *osccd7/8* mutants could be explained with positive feedback of SL deficiency on Os*D27* expression. The authors could not explain the mechanism by which D27 controls ABA levels in rice. The in vitro experiment ruled out the possibility that D27 is directly involved in forming intermediates in ABA biosynthetic pathway (9'-*cis* violaxanthin or 9'-*cis*-neoxanthin) from their all-*trans* precursors [88]. In arabidopsis, AtD27 has two closely related homologs, D27-LIKE1 and D27-LIKE2, which might also be involved in β-carotene isomerization [89, 90]. Plants with a mutation in *D27-LIKE1* gene do not present phenotypes typical for SL-depleted or SL-insensitive mutants. However, the overexpression line (*OE-D27LIKE1*) in the background of the *d27* mutant restored the more-branching phenotype, indicating the participation of AtD27-LIKE1 in SL biosynthesis [90]. More importantly, the in vitro assay showed that D27-LIKE1 displayed an affinity for all-β-carotene isoforms and accepted zeaxanthin and violaxanthin as substrates, showing that D27-LIKE1 might affect both ABA and SL content [89]. It was proposed that D27/D27-LIKE1 might indirectly control the relationship between SL and ABA biosynthetic pathways. In line with this suggestion is a study showing increased ABA concentrations in 6-week-old leaves of transgenic barley with *HvD27* gene under arabidopsis promoter AtD27 (*pAtD27::HvD27*) [91]. Moreover, the *atd27* mutant showed about 20% less ABA in shoots than WT [82]. Noteworthy, the researchers did not detect a significant difference in root samples both in rice and arabidopsis. The analysis of the overexpression of other genes involved in SL biosynthesis was also investigated regarding ABA accumulation. The increased shoot ABA levels were observed in arabidopsis transgenic lines overexpressing the soybean (*Glycine max*) orthologs of *AtCCD7*, *AtCCD8* and *AtMAX1* genes [92] (Supplementary Table 1). Thus, enhanced production of SL seems to promote ABA content in the shoot. On the other hand, the same research revealed that mutation in one of the arabidopsis *AtCCD7*, *AtCCD8* or, *AtMAX1* genes results in decreased ABA content. This observation is in contrast to rice studies [87] therefore, the role of particular genes involved in SL biosynthesis pathway remains elusive and requires further in-deep investigations both in monocots and dicots species.

Despite numerous studies indicating the mutual promotion of SL and ABA biosynthesis, scientists also indicated a possible antagonistic effect on the production of both phytohormones. In mature barley roots, elevated ABA levels by RNAi-mediated down-regulation of two ABA catabolic genes coding ABA 8'-hydroxlase (*HvABA8'OH-1* and *HvABA8'OH-3*) resulted in lower amounts of *HvD27*, *HvCCD7*, *HvCCD8*, and *HvMAX1* transcripts in two independent transgenic lines (LOHi236 and LOHi272). The limited synthesis of SL contributed to the high-tillering phenotype of RNAi mutants, suggesting that in WT plants, the homeostasis between ABA and SL is essential for controlling the tiller formation [91]. The negative impact of elevated ABA concentration on SL biosynthesis genes expression was also proved in 2-week-old rice seedlings. Application of ABA strongly repressed expression of *OsCCD8* and *OsD27* genes in roots 3, 6, and 12 h after treatment and moderately reduced *OsCCD7* expression after 12 h. Consistent with the inhibition of SL biosynthetic by ABA, expression of SL repressor *OsD53* was also significantly reduced 6 and 12 h after ABA teratment [80]. On the other hand, the negative impact of SL treatment on ABA content was also detected. In the germination assay of *Pelipanche ramosa* parasitic seeds, it is hypothesized that GR24 stimulate the ABA degradation by strongly up-regulating the *PrABA8'OH-1* gene, thereby promoting seed germination [93]. Another study corroborated this discovery, showing that the application of GR24 decreases the promoter DNA methylations of this ABA catabolic gene, promoting its expression [94]. Thus, it may be assumed that SL found in root exudates of hosting plants are a germination signal for parasitic seeds and promote their germination by degradation of ABA. Finally, the application of *rac*-GR24 markedly inhibited the ABA-induced accumulation of sugars and anthocyanins in *Vitis vinifera* (grape) berries attached to plants [95]. To summarize, the data collected indicate that changes in SL and ABA levels in plants are influenced by several factors, including the organ type and the

stage of the plant's life cycle, under ideal growth conditions. The interaction between SL and ABA can either promote or hinder the production of each other, resulting in a balance of both phytohormones and triggering an unprecedented plant response.

Interplay in SL and ABA biosynthesis pathways under abiotic stresses

Abiotic stresses such as drought, salinity, extremes of temperatures, or nutrient starvation pose a severe threat to plant growth and development, reflected in worldwide crop losses and threatening food security [96, 97]. Therefore, designing new strategies to enhance plants' adaptation to harsh circumstances is crucial. One promising approach is to comprehensively understand the phytohormone biosynthetic pathways, which play a key role in regulating plant responses to environmental stresses [98, 99]. Undoubtedly, the most well-known hormone involved in plant responses to various abiotic stresses is ABA, referred to in the literature as the stress hormone [100]. ABA rapidly accumulates to high levels during unfavourable environmental conditions, such as water deficit, soil salinity and osmotic stress, which alters the expression profile of TFs and related stress-responsive genes [101]. On the other hand, more and more research studies have evidenced a clear-cut role of SL in conferring abiotic stress tolerance across plant species.

It was shown that SL application improves the resistance of WT plants to drought stress in arabidopsis [61], wheat [77], maize [78], lettuce (*Lactuca sativa*), and tomato [79]. What is more, 3-week-old rice seedlings harbouring the mutation in the *T20* gene, which results in both lower SL and ABA concentrations, were much more sensitive to various types of stresses (osmotic stress, salt stress, dehydration, and cold tolerance) than WT plants [80]. Considering all these facts, researchers are targeting SL and ABA cooperation in abiotic stress resistance plants' mechanisms. Using the parameter of 50% inhibition of seed germination by thermo-inhibition (TI_{50}) it was shown that arabidopsis *max1* and *max2* mutants are 3 °C more sensitive to temperature than WT seeds. The application of *rac*-GR24 increased the TI_{50} of WT, *ccd7* and, *max1*, but not *max2*, revealing that hypersensitivity to heat stress is SL-dependent [102]. The effect of rescuing the phenotype of high temperature-sensitive seeds by *rac*-GR24 application was possible due to decreasing the ABA\GA ratio via suppression of heat-induced ABA increase. The lower ABA content triggered by SL was due to the inhibition of *NCED9* gene expression [102] (Supplementary Table 2), which is considered a key player in the control of seed germination and thermo-inhibition [103, 104]. It seems that the application of SL may restrict the inhibition of seeds germination in heat stress by limiting the ABA biosynthesis. Recently, the work of Chi and colleagues has shed new light on the relationship between the SL and ABA biosynthetic pathways in tomato plant responses to extreme temperature changes at the seedling phase. Exposure to 4 or 42 °C temperatures contributed to a significant upregulation of *CCD7*, *CCD8 and MAX1* genes in WT's roots and leaves. Moreover, the number of transcripts detected was intrinsically higher in the roots than in leaf samples [105]. The pre-treatment of WT and *Slccd7* plants with $GR24^{5DS}$ reduced sensitivity to heat stress, as evidenced by less serve wilting, lower relative electrolyte leakage values and malondialdehyde contents in the leaves of pre-treated plants compared to control plants. Further, SL-mediated extreme temperatures tolerance was revealed to be associated with the escalation of *NCED6* gene expression in tomato shoots, followed by increased ABA content in WT and *cdd7* tomato mutant. Moreover, the transcripts level was always lower in the mutant than in WT plants [105] (Supplementary Table 2). The opposite SL-ABA interactions were perceived with other SL biosynthesis mutants in monocotyledonous plants. Rice *d27* mutant seedlings display significantly decreased shoot ABA contents with lower transcripts amounts of ABA-responsive genes *MYB DOMAIN PROTEIN 2* (*MYB2*) and *RAB16C* and impaired cold tolerance abilities [80] (Supplementary Table 2). As the *D27* gene acts upstream of the *CCD7* gene in the SL biosynthesis pathway, the observed differences may result from the proposed role of the *D27* gene as a point connecting the SL and ABA biosynthetic pathways. This demonstrates that SL may modulate the ABA biosynthesis, influencing the ABA-dependent transcriptional responses during heat or cold stress conditions. Importantly, $GR24^{5DS}$ treatment cannot rescue the severe wilting phenotype of ABA-deficient *notabillis* tomato plants under heat and cold stresses. What is more, the SL-induced activation of extreme temperatures resistance factors (*HEAT SHOCK PROTEIN 70 [HSP70], C-REPEAT BINDING FACTOR 1 [CBF1]*) was abolished in *notabillis* plants [83]. These indications prove that SL positively regulate tomato's tolerance for heat and cold stresses in an ABA-mediated way. Hence, exogenous treatments or transgenic approaches for higher SL bioaccumulation may be potential strategies for developing tolerance to extreme temperatures in crops. However, it seems possible that the balance in ABA and SL levels may depend on the type of abiotic stress the plant is subjected to. For instance, Liu and coworkers showed that PEG-induced osmotic stress led to enhanced ABA accumulation in both shoot and roots of *Lotus japonicus*, while during the phosphate (Pi) starvation, ABA level remains untouched [106]. In contrast, SL biosynthesis is typically promoted while Pi deficiency occurs [107, 108]. Nonetheless, further research revealed that the simultaneous osmotic stress and Pi deficiency increased

ABA accumulation in both *L. japonicus* organs. This could explain why increased amounts of SL under Pi deficiency even more intensify ABA biosynthesis [106]. An SL-deficient *Ljccd* RNAi line was subjected to soil Pi deficiency stress or in combination with osmotic stress to verify this hypothesis. Plants with a silenced expression of SL biosynthesis gene did not display remarkable differences in ABA concentrations in roots compared to WT genotype under Pi starvation. In contrast, surprisingly, an upregulation in ABA metabolism was noted in shoots and roots under combined stresses, compared to Pi starvation alone. Additionally, in the pre-treated roots with *rac*-GR24, ABA level persists low despite PEG (Supplementary Table 2). All the outcomes suggest that a limitation in SL production in the roots might be necessary to allow organ-dependent ABA production (Fig. 3). Actually, *LjNCED2* gene expression in WT escalated over time the PEG treatment, while the other genes from the NCED family were unaltered [106]. The discovery that *rac*-GR24 can inhibit upregulation of *LjNCED2* suggests that particular genes from the ABA biosynthesis pathway may be SL-sensitive during specific abiotic stresses. Similar observations were noted for two identified homologues *CCD8* homologues in tobacco (*NtCCD8A* and *NtCCD8B* – both biologically active) and their changes in the expression level after the ABA treatment or under the Pi starvation [109]. The Pi deficiency caused the increase in the transcripts level in both of the analyzed genes in root tissue, but the expression of *NtCCD8A* gene was six-fold higher than that of *NtCCD8B*. However, six hours after applying ABA, a three-fold increase in *NtCCD8B* transcripts level was detected, whereas *NtCCD8A* transcript levels were maintained. Obtained results suggest that different genes from the SL biosynthesis pathway may be regulated either by ABA levels or/and depend on the type of abiotic stress. Based on the relationships presented above, it appears reasonable to supplement the analyzes of SL/ABA accumulation in response to various abiotic stresses with an examination of the relative expression or mutations in the individual genes involved in hormone biosynthesis. However, also in this area of research, some inaccuracies may arise. The RT-qPCR analysis showed that rice *NCED1* gene expression in both drought tolerant and drought susceptible cultivars was progressively reduced with increasing water withholding stress, simultaneously with increasing ABA content [64]. In contrast, reports in other species like tomato [110] and barley [111] have evidenced that *NCED1* transcripts level is higher under drought stress than under control conditions. It would be interesting to detect if the function of individual genes in the *NCED* family may be species-dependent.

During the salt stress conditions, another player that may mediate the SL-ABA biosynthesis pathways interactions was revealed. Under control conditions, the expression of *CCD7* and *CCD8* homologues in arbuscular mycorrhizal (AM) *Sesbania cannabina* seedlings roots increased significantly after the ABA treatment and more interesting after the hydrogen peroxide (H_2O_2) application [112]. Similar observations were noted under salt stress conditions, where both SL-biosynthesis genes' expression increased multi-fold. Furthermore, the germination assay of *P. ramosa* seeds induced by AM *S. cannabina* seedling root extracts revealed that under stress conditions, ABA-induced SL production was inhibited by a pre-treatment with dimethylthiourea (DMTU), which scavenges H_2O_2. On the contrary, ABA accumulation remains unaffected by DMTU. Hence, ABA appears to function upstream of H_2O_2 in ABA-induced SL accumulation in AM *S. cannabina* seedlings. Additionally, *rac*-GR24 contributed to rescuing the salt stress tolerance in the ABA-deficient plants. In contrast, ABA could only partially rescue the impaired salt stress tolerance in plants treated with tungstate (SL biosynthesis inhibitor) [112]. All this implies that ABA and SL work together to maintain salt stress tolerance in *S. cannabina* seedlings by ABA – H_2O_2 – SL pathway. Cooperation between SL and ABA biosynthesis pathways in salt stress was also noted in arabidopsis [60] and lettuce plants [113]. Most studies investigating the relationship between the ABA and SL biosynthetic pathways are related to drought stress however, current researches show many discrepancies. Water scarcity contributes to the activation of various defense mechanisms aimed at water retention in cells and organs. One of the best-known effects of plants against water loss is limiting transpiration by closing the stomata. This process is controlled by ABA, whose levels increase rapidly during drought stress. Moreover, the expression of SL biosynthesis genes in shoot also increased multi-fold times, followed by enhanced phytohormone accumulation in plants tissues, noted in several plant species, including arabidopsis [61] and tomato [93] (Fig. 3). In addition, plants harbouring mutations in the *CCD7* or *CCD8* genes display decreased drought tolerance due to ABA hyposensitivity at the guard cell level [61, 79, 84, 93]. However, the published results of experimental work aimed to determine the function of SL under water deficiency in arabidopsis were contradictory [83, 114]. While a slightly different experimental setup might explain some inconsistencies (different growth conditions, seedling age, and different periods of exposure to drought), it is puzzling that in one instance, SL biosynthesis mutants presented drought-sensitive phenotype [83], while in the other, their behaviour did not differ from the WT [114]. Ha and coworkers proved their results by hormone treatment of SL-depleted mutants and WT plants, rescuing the drought sensitive phenotype or enhancing the stress tolerance, respectively [83] (Supplementary Table 2).

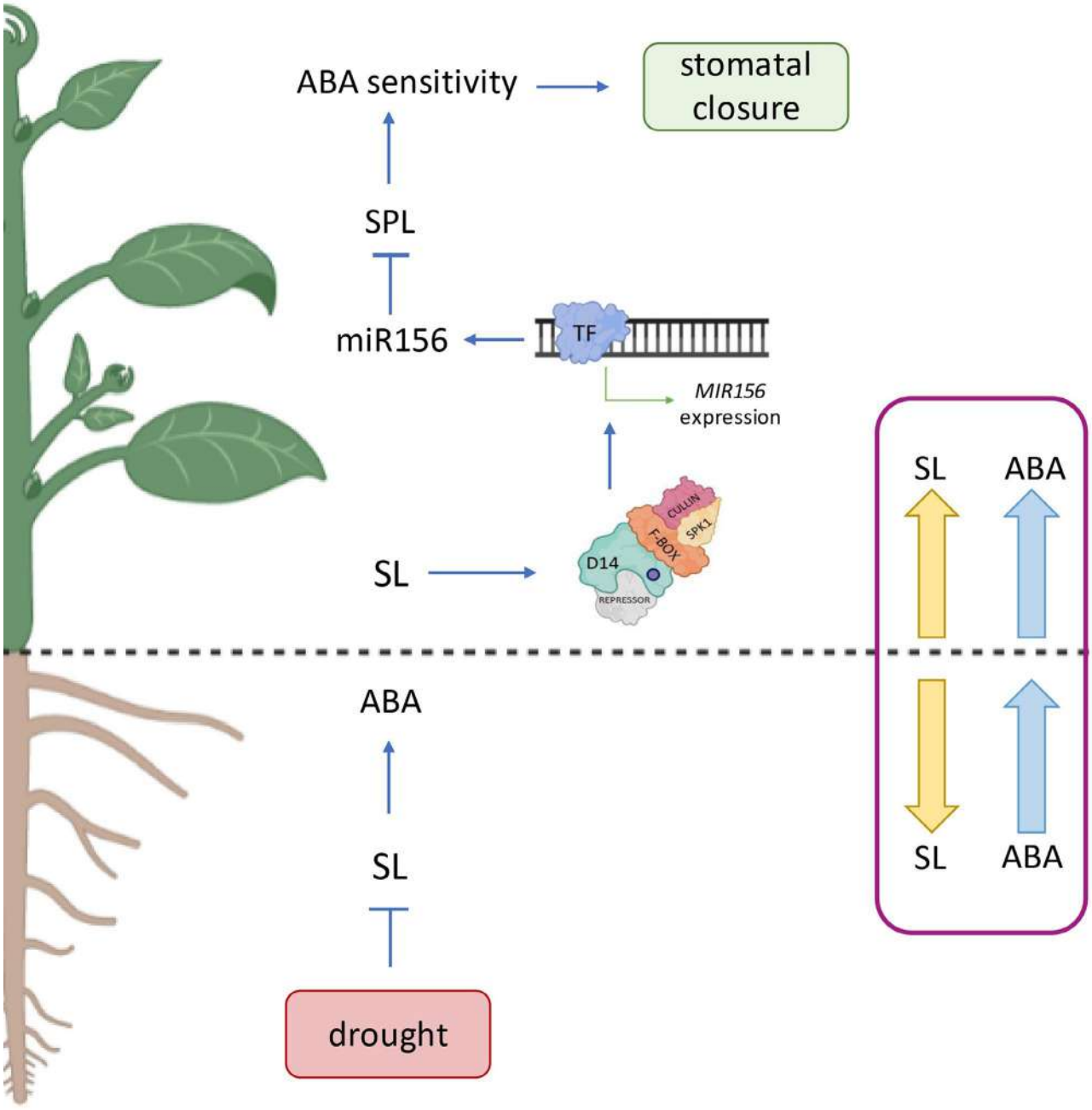

Fig. 3 The model of ABA and SL organ-specific relations under drought. In the below-ground organ part of plants the relations between SL and ABA are concentrated on the biosynthesis level. The drop of SL content in roots plays a sensor role of plant stress and promote ABA accumulation, thus activating the plant resistance mechanisms. In the shoots, enhanced SL biosynthesis leads to the degradation of SL repressor through the assembly of the D14-SCF complex. This, in turn, activates the expression of the *MIR156* gene, resulting in the accumulation of mature miR156 molecules that inhibit mRNA translation. This process ultimately prevents the formation of SPL native proteins, making guard cells more sensitive to ABA and accelerating their closure. The blue and yellow arrows indicate the content of ABA or SL in each plant organ during drought stress. D14 – DWARF 14, SCF – SKP1-CULLIN-F-BOX, TF – transcription factor, SPL - SQUAMOSA PROMOTER BINDING PROTEIN-LIKE. Created with BioRender.com

On the other hand, a study conducted on rice complements the presented issue and aligns with the results obtained by Bu and colleagues. Here, *CCD7* and *CCD8* rice mutants showed significantly higher survival rates than WT under drought stress [87]. Also, in support of this view, researchers detected enhanced ABA accumulation in the shoots of SL-depleted (*CCD7* and *CCD8*) rice plants, resulting in more efficient water retention because of accelerated closing of the stomata. In contrast rice *d27* mutant was unable to survive under the same drought

conditions. Additionally the ABA levels in *d27* mutants were also lower than in the WT plants under drought [87]. The above-mentioned inconsistencies in the tolerance of SL-biosynthesis mutants to drought conditions may result from the different production of SL in monocotyledonous and dicotyledonous plants.

Cross-talk between SL and ABA pathways at the perception level

Crosstalk between SL- and ABA-related genes is associated with the balance of endogenous hormones level, but also with changes in the sensitivity of plants to hormone presence. The fact that SL-depleted plants are hypersensitive to various environmental stresses and hyposensitive to ABA in the aspect of stomatal closure was corroborated in three genetically distant plant species, arabidopsis [61], tomato [93], and *L. japonicus* [84], by independent research groups. Therefore, it is also sufficient to elaborate on the relationship between SL and ABA signaling pathways. So far, little research has addressed the SL-ABA interplay at the signaling level under abiotic stress conditions. One of the presented issue's first studies was carried out on arabidopsis F-box protein from the SCF complex – the *MAX2* gene [83, 114]. Two independent groups presented a novel function of the *MAX2* gene in plant drought response, expanding its role in an ABA-dependent manner. Arabidopsis *max2* mutant is hypersensitive to drought and evaporates more water than WT plants due to a thinner cuticle layer, increased stomatal density and inefficient stomatal closure caused by lower responsiveness to ABA [83, 114]. What is more, the qPCR analysis reveals that the relative transcripts level of ABA signaling, biosynthesis, transport, and catabolism genes were diminished in *max2* compared to WT seedlings under drought conditions [114] (Supplementary Table 3). In general, presented observations indicate that crosstalk between SL and ABA is prominent in the transduction of stress signals. However, the analyzes carried out on mutants in the genes encoding the F-box protein from the SCF complex (*AtMAX2*/*OsD3*) in terms of the functioning of the SL signaling pathway seem controversial due to the participation of these the F-box proteins in the signal transduction pathway of KAR [115], which engagement in drought stress tolerance was also elaborated [116].

Another experimental examined component from the SL-signaling complex in terms of ABA-related drought response is SL-repressor. It is expected that mutation in the SL-repressor should have the opposite effect on plant functioning to the SL-depleted or SL-insensitive plants due to the constantly active SL transduction pathway. In arabidopsis genome, three genes encoding SL repressors have been identified so far – *SMXL6*, *SMXL7* and *SMXL8* [117]. Characterizing single and double mutant combinations under drought stress revealed that knockout of one of the SL-repressor genes makes no difference in the plant survival rate compared to WT, while mutations in two SMXL genes cause mild promotion of drought resistance [118]. The two different triple *smxl6/7/8* mutant lines exhibited significantly higher drought tolerance than WT (Supplementary Table 3). All these facts clearly highlight the functional redundancy of SMXL6,7,8 proteins acting as negative transcription regulators of SL signaling in arabidopsis. The increased drought tolerance of triple mutant was investigated in detailed physiological and biochemical analysis. Reduced cuticle permeability, increased anthocyanin biosynthesis, enhanced reactive oxygen species (ROS) detoxification capacity, and decreased water loss were detected, which might help *smxl6,7,8* mutant plants survive drought [118]. Additionally, the authors recorded higher expression levels of ABA INSENSITIVE 5 (ABI5) and SENESCENCE-ASSOCIATED GENE 29 (SAG29) genes after 2 and 4 h of dehydration in *smxl6,7,8* mutant than in WT plants. Both of these genes have been widely used as a marker gene for ABA response, thus suggesting that the increased tolerance of *smxl6,7,8* plants might be connected with ABA hypersensitivity. Notably, the increased sensitivity to ABA of the triple mutant compared to WT was also proved in both cotyledon opening and growth inhibition assay [118]. Analogous observations were noted in the case of arabidopsis plants harbouring a mutation in *SUPPRESSOR OF MAX2 1* (*SMAX1*) and *SMXL2* genes. SMAX1 and SMXL2 are components of the core signal transduction complex of the KAR, suppressing the activity of MAX2, which is a common point in both KAR and SL signaling pathways [119]. The *smax1/smxl2* mutant exhibited enhanced drought tolerance due to increased cuticle formation and ABA hypersensitivity, which was proved in assays of stomatal closure, cotyledon opening, chlorophyll degradation, and growth inhibition [120]. Since not all SL signaling transduction pathway components are SL-specific [115], it was postulated that mutants in the SL receptor D14 should be considered a gold standard in studies disclosing the role of SL in plants [121]. Barley *hvd14.d* mutant displayed hypersensitive to drought phenotype, illustrated by lower leaf relative water content (RWC), impaired photosynthesis, disorganization of chloroplast structure, altered stomatal closure and density [121] (Supplementary Table 3). The transcription profile of ABA signaling genes, including *HvPYL4*, *HvPP2C4*, *HvSnRK2.1* and *HvABI5* remain unchanged in *hvd14.d* mutant compared to WT under drought stress [121]. On the other hand, the expression of genes related to ABA biosynthesis, such as *HvNCED1*, *HvNCED2*, and *HvAo5b* was up-regulated in the mutants due to water deficit. The outcomes suggest that the mutant's drought tolerance reduction is probably

caused by an inability to respond to the elevated ABA levels and trigger a proper stress response [121]. Hence, it can be assumed that SL-insensitive plants show reduced ABA signal perception. Additionally, drought-sensitive phenotype and physiological deterioration caused by stress were also proved in the same research on arabidopsis *atd14-1* plants. The same plant drought hyposensitivity phenotype as in the case of *hvd14.d* and *atd14-1* was noted during independent research focused on *atd14-2*. In this study, loss-of-function of the *D14* gene was associated with lower anthocyanin content, delayed senescence, and slower ABA-mediated stomatal closure [122]. Overall, mutants in the SL biosynthetic and SL signaling genes have been shown to have a higher stomatal conductance than the WT in the presence or absence of abiotic stresses and an impaired response to ABA treatment [83, 87, 106, 114, 118, 121, 123]. Therefore, the participation of SL in proper guard cell functioning and adjusting plant responses to water deprivation is supported enough to consider SL as a crucial factor in determining the plants' drought tolerance. Especially since the expression of *MAX2* and *D14* genes are wide and more enriched in the stomatal lineage than in other leaf tissue [124]. In addition, the simultaneous application of ABA and *rac*-GR24 resulted in a smaller diameter of stomata than that of ABA or *rac*-GR24 alone [124] (Supplementary Table 3).

Recently it was shown that treatment with $GR24^{5DS}$ contributes to increasing plant's drought tolerance by efficient stomata closure, followed by enhanced accumulation of *miR156* molecule in tomato leaves [125]. To date, several studies indicate the role of *miR156* and its targets belonging to the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) family in regulating stress tolerance [126–129]. To understand if the enhanced levels of *miR156* are a consequence of elevated SL shoot accumulation during drought, the SL-depleted plants were subjected to water deprivation. No induction of *miR156* biogenesis could be observed in *CCD7*-silenced plants under drought conditions compared to WT. Further analyses revealed that the overexpression of the *AtMIR156* gene led to higher ABA sensitivity [125]. In addition, the stomatal closure induced by ABA spraying was more pronounced in *miR156-oe* plants than in WT (Supplementary Table 3). Hence, researchers have shown that the *miR156* may be the connecting point of both ABA and SL signaling pathways in the aspect of stomata action [125] (Fig. 3). However, some studies indicate that SL may play an active role in the closure of the stomata in an ABA-independent way, which was proven in several plant species, including arabidopsis [124, 130], *Vicia faba* [131] and, grape [132]. Arabidopsis plants could close their stomata three hours after the *rac*-GR24 treatment in a dose-dependent manner [124]. In addition, the same observations were noted in the SL-induced closure of stomata in multiple various lines of ABA biosynthesis, receptors and signaling mutants. Because H_2O_2 is an essential secondary messenger in closing stomata, the participation of that molecule in SL-induced stomata responses was also investigated. Indeed, SL-induced stomata closure was utterly blocked in ascorbic acid or catalase presence, reducing the H_2O_2 amount in cells [124] (Supplementary Table 3). A similar effect was observed under the nitrogen oxide (NO) analysis, where the PTIO (an NO scavenger) and Na_2WO_4 (a nitrate reductase inhibitor) prevented SL-induced stomatal closure. Moreover, the analysis indicated that mutation in the *SLOW ANION CHANNEL-ASSOCIATED 1* (*SLAC1*) gene (a key player in ABA-induced stomatal closure) resulted in ABA and SL insensitivity, pinpointing that both hormone signaling pathways modulate the osmotic pressure by SLAC1, leading to the closure of stomata [126]. All together suggests that SL mechanisms leading to the closing of the stomata require the accumulation of both H_2O_2 and NO in the guard cells and activation of *SLAC1*, similar to ABA. Another study reveals that Ca^{2+} chelator and Ca^{2+} channel blockers strongly inhibit the SL-induced closure of stomata [130]. Through examining a collection of calcium-dependent protein kinase (CPK) mutants, the CPK33 protein was identified as a potential Ca^{2+} transducer involved in SL-mediated stomata response. The *cpk33* mutant was impaired in SL-, H_2O_2- and Ca^{2+}-induced stomatal closure. Thus researchers propose that SL stimulate the production of H_2O_2 that possibly activates the Ca^{2+} transducer CPK33 which likely modulates anion and potassium channels to promote stomatal closure. In contrast to all the presented data above, there is one study where treatments with a SL analogue cannot induce stomatal closure in arabidopsis [133] however, conductivity analysis was performed within one hour after SL treatment, which may not be sufficient time to observe a physiological effect.

Organ-specific dynamics of SL and ABA relations

The studies above clearly indicate the interaction between the ABA and SL biosynthesis and signaling pathways under control conditions and response to various abiotic stresses, especially drought or salinity. In particular, previous experimental research on arabidopsis, tomato and, *L. japonicus* allowed proposing a model connecting SL and ABA levels in a root-shoot-dependent manner during drought stress [125, 134]. In this model, the drop in SL biosynthesis in the roots may be required to empower ABA production. In this context, SL might play a sensor role in water deprivation, then promote the ABA accumulation in root tissue. Indeed, under water scarcity, ABA accumulation in root tissues, followed by increased ABA content in the shoot, is closely correlated

with a decrease in leaf stomatal conductance [135] or alleviates stress by other mechanisms [136] (Fig. 3). Referring to the presented model, it is believed that inhibited shootward flow of SL may trigger SL biosynthesis in shoots by an unknown mechanism. Especially since greater amounts of SL are produced in the roots, hormone molecules are probably more intensively transported to the shoot under optimal conditions. Under stress, the enhanced regulation of SL biosynthesis genes in the above-ground organs of various plant species may suggest that SL play an active role in overcoming harsh environmental conditions and increasing plants' survival rate. The enhanced activation of SL biosynthesis genes in shoots was proved by transcript quantification during stress in several plant species, such as arabidopsis [61], tomato [93] and rice [87]. What is more, using a reciprocal grafting approach between SL-deficient mutants and WT plants, it was demonstrated that stomatal closure is affected by the shoot genotype rather than the root genotype. WT tomato scions grafted onto SL-depleted rootstock exhibited an increased amount of SL biosynthetic genes' transcripts, as well as lower transpiration phenotype under drought compared to control grafted plants [123]. Further analysis revealed that the more efficient closure of the stomata was due to enhanced sensitivity to endogenous ABA, rather than an increase in total free ABA. Similarly, previous data related to *L. japonicus* indicate no changes in ABA accumulation in shoots of SL-depleted plants under osmotic stress compared to WT [106], which suggests that SL-ABA relations in above-ground organs might occur at the perception level. However, tomato and *L .japonicus* studies were conducted on plants harbouring the mutation in *CCD7* gene. In contrast, one research that proves that under drought stress, the mutation in *CCD7* and *CCD8* genes led to increased ABA accumulation in leaves, in opposition to *d27* mutation, where the ABA content decreased significantly compared to control plants [87]. Unfortunately, the research was carried out on rice, the monocot specie. To date, no evidence confirms a similar relationship in dicots plants during drought conditions. Therefore, the *D27* gene should be included in analysing the SL-ABA crosstalk in dicots under stress. The unchanged ABA levels compared to WT plants were also noted in barley SL-insensitive *hvd14.d* mutant under dehydration conditions [121]. A few additional players contributing to the closure of the stomata, including H_2O_2, NO, miRNA156, *SLAC1* and *CPK33* in either ABA-dependent or ABA-independent ways, were identified. It was proposed that SL may trigger the ABA sensitivity in guard cells by the interaction between miR156 and SL repressor protein [137]. Under optimal environmental conditions, the presence of SMXL6,7,8 transcriptional repressors inhibits the *miR156* biogenesis. In turn, the SPL transcription factors may accumulate, maintaining the ABA sensitivity at the low level and opening stomata. In contrast, under drought conditions, the activation of SL biogenesis, followed by assembling the SL signaling complex, leads to the degradation of SMXL6,7,8 proteins. Consequently, the *miR156* molecules may accumulate and inhibit mRNA translation, thus blocking the formation of SPL native proteins. This molecular cascade is believed to increase the sensitivity of guard cells to ABA and accelerates their closure (Fig. 3). On the other hand, combining previous research of SL-induced closure of stomata in ABA-independent way the mechanism might be based on the activation of SLAC1 by H_2O_2/NO and CPK33 pathway. It was proved that SL biosynthesis and further SL signaling lead to H_2O_2 and NO production. Next, activation of SLAC1 modulates the osmotic pressure in guard cells, leading to the closure of stomata [124]. In addition, another study revealed that CPK33 is required for SL-modulated proper stomata functioning [130]. It is important that the *cpk33* mutant is impaired in H_2O_2-induced stomatal closure, but not in SL-mediated H_2O_2 production. This clearly highlights that CPK33 acts downstream upon H_2O_2/NO in SL-induced stomata regulation. It was also shown that in arabidopsis guard cells, anion channel SLAC1 is regulated by CPK proteins [138]. Thus, the SL-induced regulation of closing the stomata under drought might be activated by SL – H_2O_2/NO – CPK33 – SLAC1 pathway (Fig. 4). It is puzzling that CPK33 was reported as a negative regulator of slow anion channels activity in ABA-induced stomatal closure [139, 140], unlike where the *CKP33* gene with mutation blocked SL-induced stomata regulation, clearly indicating the role of CPK33 as a positive SL-mediated stomatal regulator. During ABA-dependent pathway, the SLAC1 might be activated either by calcium-independent kinases, such as OPEN STOMATA 1 (OST1) or CPK proteins [141] (Fig. 4). Under water-deficit, stress can trigger ROS accumulation and promote activation of Ca^{2+} channels, resulting in increased Ca^{2+} in the cytoplasm of guard cells [142]. CPK then perceives the Ca^{2+} cations to validate signal transduction. The phosphorylation signal promotes the conformation changes of SLAC1, thus enabling the outflow of anions outside the guard cell. Further, with the outflow of cations from the cell, the ionic strength outside the guard cell increases, followed by H_2O outflow. The turgor of the guard cell decrease, which leads to stomatal closure. The role of a positive calcium-dependent kinase regulator of ABA-mediated stomata closure was experimentally proved for several CPK proteins, including CPK3/6/21/23 (Fig. 4) [143]. However, mutation of *CPK33* resulted in arabidopsis the ABA-dependent hyperactivation of SLAC1, while the *CPK33* overexpression showed opposite phenotype [139, 140]. Taken together, the CPK33 might be an essential player in both

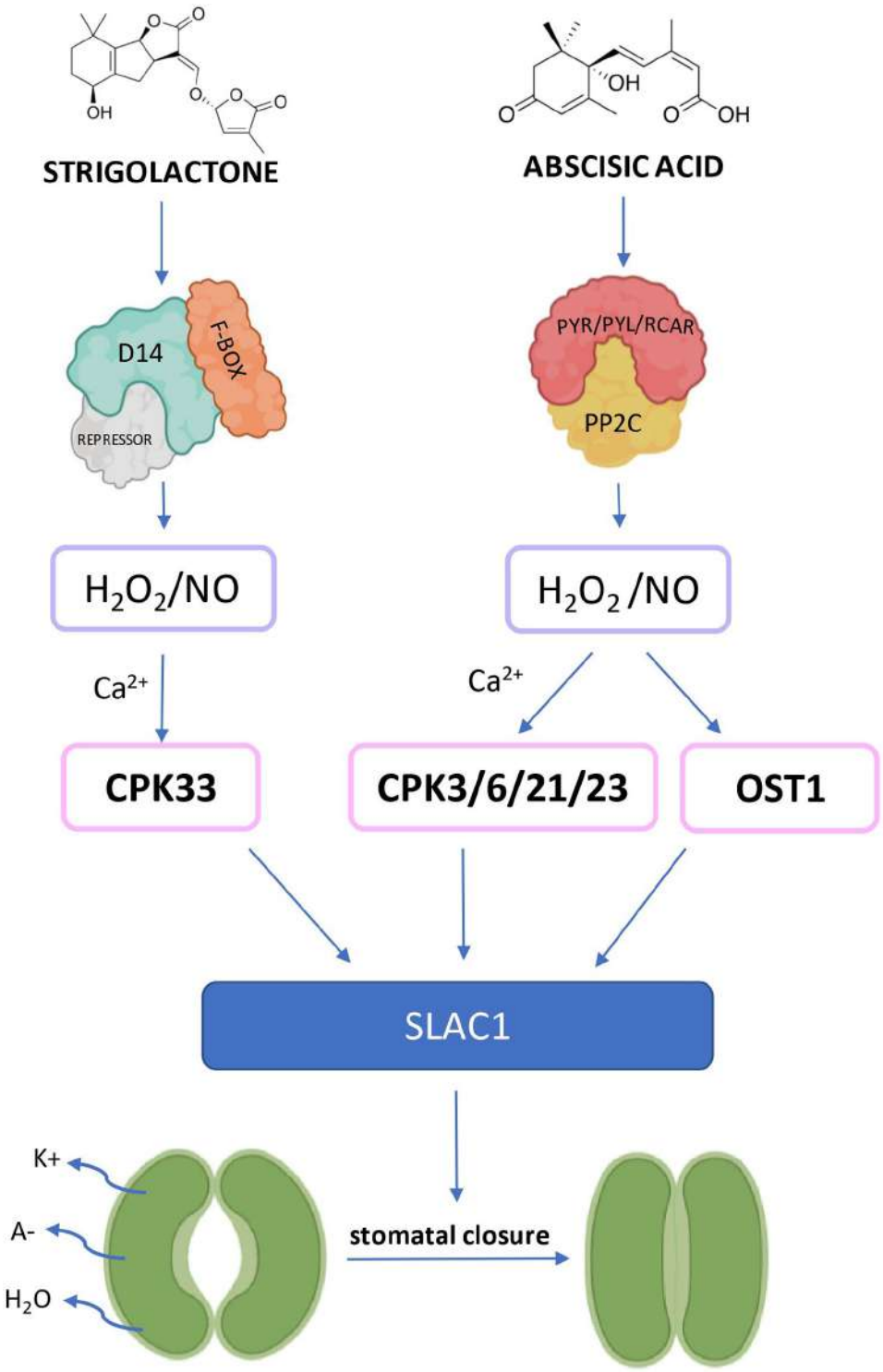

Fig. 4 SLAC1 is a common point providing stomatal closure in SL- and ABA-dependent ways. Both SL and ABA signaling pathways initiate the production of secondary messengers for stomata movement, namely H_2O_2 and NO. These molecules indirectly activate calcium-dependent (CPK) or calcium-independent kinases (OST1), which provide the phosphorylation signal promoting conformational changes of SLAC1 and outflow of anions (A-) outside the guard cell. Further, with the outflow of cations (K+) from the cell, the ionic strength outside the guard cell increases, followed by H_2O outflow. The turgor of the guard cell decrease, which leads to stomatal closure. PYR - PYRABACITN RESISTANCE, PYL - PYRABACTIN RESISTANCE 1-LIKE, RCAR - REGULATORY COMPONENT OF ABA RECEPTOR, PP2C - PROTEIN PHOSPHATASE 2 C, CPK – CALCIUM-DEPENDENT KINASE, D14 – DWARF 14, OST1 – OPEN STOMATA 1, SLAC1 – SLOW ANION CHANNEL-ASSOCIATED 1. Created with BioRender.com

ABA- and SL-dependent control of stomata closure. Nevertheless, the discrepant role of CPK33 in guard cell ABA and SL signaling is needed to be further unraveled. Presented results indicate that SL and ABA crosstalk dynamics at the biosynthesis and perception level are seemingly opposite in the above- and below-ground organs, reinforcing the need to separate roots and shoots analysis when addressing issues related to SL-ABA interactions under stress.

Main open questions and future goals

The primary hormone associated with the plant response to drought stress is ABA [144]. With an increase in experimental data indicating the participation of SL in maintaining stress tolerance, it is expected that SL might interplay, directly or indirectly, with ABA in regulating adaptive stress responses in plants. Thus, the crosstalk between SL and ABA's biosynthetic and signaling pathways during abiotic stresses is eagerly investigated. At the biosynthesis level, the SL-ABA relations in roots are pretty well documented regarding growth and developmental processes or in response to abiotic stresses. However, some inconsistencies exist in the metabolic SL-ABA interplay at the shoot level. There is an open question if SL may trigger ABA biosynthesis in response to drought or whether the SL-ABA crosstalk is related only to perception level. Beyond the above observations, which suggest that the influence of SL and ABA on their mutual concentrations may be more or less intimate in different species and organs, more and more research is focusing on the crosstalk between the signaling pathways of both hormones. First, the mechanism underlying root-to-shoot communication at the SL level requires in-depth investigation. It is tempting to see how the decreased levels in roots might contribute to the activation of SL biosynthesis in leaves. Finally, it would be interesting to experimentally confirm the relations between SL-repressor and *miR156* leading to enhanced ABA sensitivity, as was recently proposed [137].

List of abbreviations

AAO	Abscisic acid oxidase
ABA	Abscisic acid
ABA-GE	ABA-glucosyl ester
ABI	Abscisic acid insensitive
ABRE	ABA-responsive element
AM	Arbuscular mycorrhizal
AUX	Auxins
BR	Brassinosteroids
BRC	Branched
CBF	C-repeat binding factor
CCD	Carotenoid cleavage dioxygenase
CKs	Cytokinins
CLA	Carlactonoic acid
CLAMT	Carlactonoic acid methyltransferase
CPK	Calcium-dependent protein kinase
D	Dwarf
DAD	Decreased apical dominance
DMTU	Dimethylthiourea
ET	Ethylene
GA	Gibberellins
HSP	Heat shock protein
HTD	High-tillering dwarf
JA	Jasmonates
KAI	Karrakin insensitive
KAR	Karrikins
MAX	More axillary growth
MeCLA	Methyl carlactonoate
MYB	Myb domain protein
NCED	Epoxycarotenoid dioxygenase
NO	Nitrogen oxide
NSY	Neoxantin synthetase
OST	Open stomata
Pi	Phosphate
PAP	Production of anthyocyanin pigment
PEG	Polyethylene glycol
PP2Cs	Protein phosphatase 2 C
PYL	Pyrabactin resistance 1-Like
PYR	Pyrabactin resistance
RCAR	Regulatory component of aba receptor
RMS	Ramousus
RWC	Relative water content
SCF	SKP1-Cullin-F-Box
SLAC	Slow anion channel-associated
SL	Strigolactones
SMAX	Suppressor of Max2
SMXL	Supressor of Max2 1-Like
SnRK2s	Sucrose nonfermenting 1 related protein kinases 2
SPL	Squamosa promoter binding protein-like
T	Tillering
TCP	TCP domain protein
TF	Transcription factor
TI_{50}	50% inhibition of seed germination by thermo-inhibition
UPS	Ubiquitin proteasome system
WT	Wild type
XD	Xanthoxin dehydrogenase
ZEP	Zeaxanthin epoxidase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-023-04332-6.

Supplementary Material 1: Supplementary Table 1: SL-ABA under control conditions. Table summarizing interactions of SL-ABA biosynthesis under control conditions.

Supplementary Material 2: Supplementary Table 2: SL-ABA biosynthesis under stress conditions. Table summarizing interactions of SL-ABA biosynthesis under stress conditions.

Supplementary Material 3: Supplementary Table 3: SL-ABA percepcion under stress conditions. Table summarizing interactions of SL-ABA signaling under stress conditions.

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Authors' contributions

M.K.: writing – original draft preparation (creation and/or presentation of the published work, specifically writing the initial draft), visualization (preparation and creation of the published work, specifically visualization/data presentation), conceptualization (ideas; formulation or evolution of overarching research goals and aims)M.M.: conceptualization (ideas; formulation or evolution of overarching research goals and aims), writing – review & editing (preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre- or postpublication stages), funding acquisition (acquisition of the financial support for the project leading to this publication).

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Data Availability
All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

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The authors declare no competing interests.

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CHAPTER

An update on strigolactone signaling in plants

4

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1. Diversity of signaling pathways among plant hormones

Since the discovery of the first plant hormone, nearly a century of research has brought extensive knowledge about phytohormone biology, including the molecular mechanisms of phytohormone perception and signaling. In general, plant hormone signaling pathways can be divided into three main categories: 1/F-box mediated signaling pathway; 2/two-component system; and 3/phosphorylation/dephosphorylation signal relay (Fig. 4.1). The vast majority of phytohormone signaling pathways rely on the repressor degradation mechanism, named as F-box-mediated signaling pathway (Aziz et al., 2022). The critical element in controlling the expression of downstream plant hormone-responsive genes is the F-box protein, which is part of the SKP-cullin-F-box (SCF) complex (Skaar et al., 2013). The F-box protein targets the repressor via ubiquitination, and next the repressor is degraded by the 26S proteasome. Thus, the interaction between the F-box protein and the repressor determines the specificity of this system. It is predicted that nearly 700 F-box proteins are in Arabidopsis (*Arabidopsis thaliana*) proteome, indicating that plants may assemble a great number of functional SCF complexes, possibly controlling the hundreds of different pathways (Bla´zquez et al., 2020). The proteolytic targets in these transduction mechanisms function as transcriptional regulators that do not directly bind to DNA. Instead, the repressors control the expression of downregulated genes by interacting with DNA-binding transcription factors (TFs). This mode of action characterizes the auxin, gibberellins, jasmonates, karrikins, and strigolactones signaling pathways (Abd-Hamid et al., 2020). All of them are indicated by a rapid response, with the target protein degradation occurring within minutes after hormone treatment (Gray et al., 2001; Guo and Ecker, 2003; Larrieu et al., 2015; Zenser et al., 2001; Zhou et al., 2013). Another well-understood signal transduction cascade is a two-component system, whereby a histidine kinase protein (HK) acts as a transmembrane-localized receptor and transmits a phosphor-relay signal to histidine phosphotransfer protein, which then phosphorylates the response regulators (Fig. 4.1) (Bowman et al., 2019). The response regulator protein (RRP) can then serve as a positive (type-B response regulator, RRB) or a negative (type-A response regulator, RRA) factor that affects the expression of target genes (Müller and Sheen, 2007). The RRP

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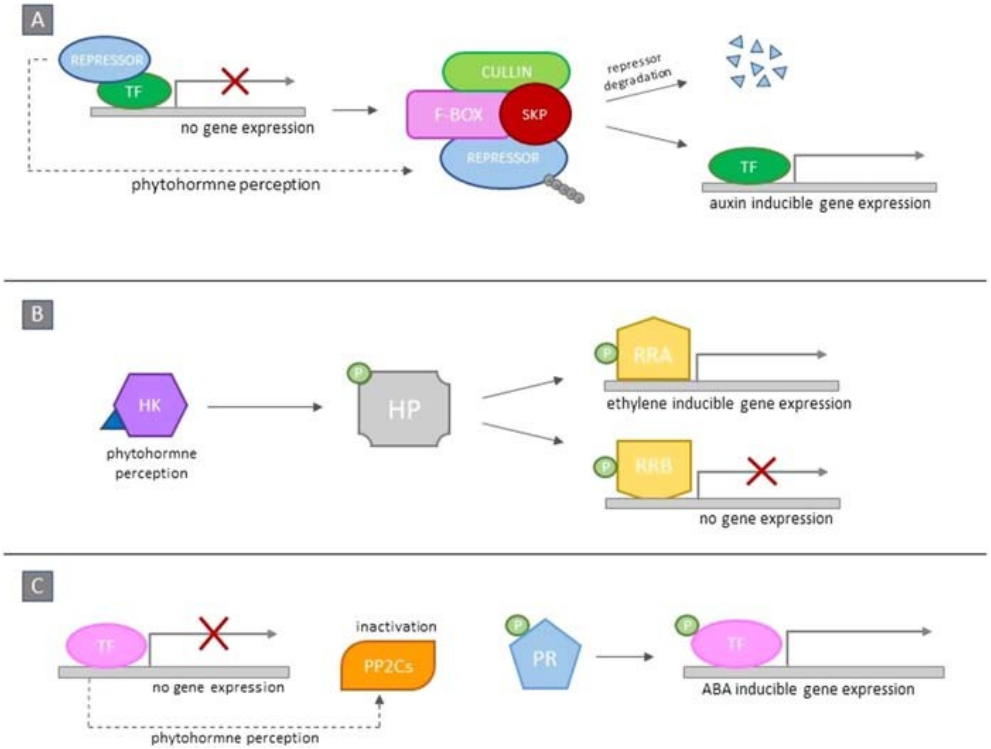

FIGURE 4.1

Scheme of the diversity of plant phytohormone signaling pathways representing three main categories: (A) F-box-mediated signaling pathway, (B) two-component system, (C) phosphorylation/dephosphorylation signal relay. Each category is visualized using one of the signaling pathways as an example, including auxin, ethylene and abscisic acid (ABA) transduction, respectively. (A) After the phytohormone perception, the SKP-cullin-F-box (SCF) complex is assembled with an F-box protein. The F-box protein targets the repressor via ubiquitination, and next the repressor is degraded by the 26S proteasome, thus releasing the transcription factors (TF). (B) Phytohormone molecule is perceived by a histidine kinase (HK) protein, which promotes the phosphorylation of histidine phosphotransfer (HP) protein. The HP transmits the phosphate residue to the type-A response regulator (RRA) or type-B response regulator (RRB), resulting in transcription regulation. (C) The phytohormone perception by ABA receptor inactivates the protein phosphatase 2C (PP2C) proteins, thus promoting the autophosphorylation of positive regulators (PRs). The activated PR transmits the phosphorylation signal to TF, which promotes the ABA inducible gene transcription.

consists of two functional domains in the N- and C-terminus regions of the proteins. The N-terminus domain possesses a conserved aspartic acid residue that serves as an acceptor site for phosphorylation during the multistep phosphorelay. The C-terminus site of RRP varies in length, but all have a conserved DNA-binding

domain (Rashotte, 2021). The His-to-Asp system is well reflected in cytokinin (Kieber and Schaller, 2018) and ethylene (Binder, 2020) signaling pathways. Although ethylene signal transduction is much more complex and requires additional functional proteins, including SCF complex, this pathway is also comprised in the two-system component category due to the initial signaling steps. It is divergent that the HK receptor of ethylene signal acts as a negative regulator, thus the ethylene perception leads to blocking of the phosphor-relay and cleavage of the RRB protein (Binder, 2020; Park et al., 2023). The C-terminus site of RRP protein dissociates the TF from the SCF complex activating the transcription of downstream genes. The TF in ethylene signaling pathway is constitutively expressed; however, it is unable to accumulate because of permanent degradation by the 26S proteasome (Zhao et al., 2021). Hence, the target of SCF-mediated proteolysis in ethylene signal transduction and other F-box plant hormones signaling pathways remain different. The SCF complex attacks the transcription activator or repressor in the molecular cascades of ethylene, auxin, gibberellins, jasmonate, strigolactones, and karrikins, respectively. The abscisic acid and brassinosteroids signaling pathways represent the third category, based on phosphorylation/dephosphorylation reactions (Fig. 4.1). However, mechanisms leading to the expression of their responsive genes are opposite. The signal perception by abscisic acid receptor inactivates the repressor (protein phosphatase 2C, PP2C) and promotes the autophosphorylation of abscisic acid positive regulators, resulting in phosphorylation of TFs and activation of downstream genes expression (Cardoso et al., 2020). In contrast, the bounding of brassinosteroids molecules by the brassinosteroids receptor leads to the mutual phosphorylation of the receptor and its coreceptor, which inactivates the negative regulator (Planas-Riverola et al., 2019). Then, the transduction cascade is triggered, resulting in dephosphorylation of another downstream suppressor; thus, the positive regulators (protein phosphatase 2A, PP2A) are allowed to dephosphorylate the TF and modulate the plant's response.

Basically, the regulation of phytohormone downstream gene expression is conditioned by the TF binding to precise nucleotide sequences, termed response elements (REs), in the promoter of those genes (Lieberman-Lazarovich et al., 2019). Binding between TF and RE regulates the transcription of genes whose protein product is necessary at a given moment (Pruneda-Paz et al., 2014). Therefore, identifying cis-elements associated with a plant's response to a specific phytohormone is essential to elucidate the mechanisms that drive plant development. In *Arabidopsis*, transcriptional regulation is mediated by approximately 1500 TF, which controls multiple genes' expression, in a complex signaling network (Riechmann et al., 2000). It is prominent that phytohormone signaling pathways are not linear and isolated cascades. In fact, vegetative, generative, and plant defense processes depend on the interaction, both antagonistically and synergistically, between phytohormones at biosynthesis and signaling levels (Berens et al., 2017). These multilevel and multicomplex phytohormone cross-talk determines the outcome of downstream responses activated in plants (Altmann et al., 2020). For example, analyses of depleted or insensitive mutants, supported by exogenous hormone applications, have shown

that both auxin and strigolactone modulate each other's accumulation for coordinated shoot branching (van Rongen et al., 2019). In addition, plant defense against pathogens requires the interaction of jasmonic acid and ethylene. Under abiotic stress conditions, the jasmonic acid and ethylene metabolic pathways synergize to activate specific gene sets (Zhu and Lee, 2015). Finally, it is well known that abscisic acid and gibberellic acid act antagonistically during seed germination (Liu and Hou, 2018). These interactions highlight the importance of a deep understanding of the plant hormone signaling pathways as a complex network. To achieve this goal, it is necessary first to thoroughly understand the signal transduction mechanism of each phytohormone separately. This chapter presents the current state of knowledge regarding strigolactone signaling.

2. Strigolactone perception

Phytohormone perception is based on the ligandereceptor interaction, in which the receptor recognizes and binds the ligand (phytohormone molecule) (Chow and McCourt, 2006). Up to now, only one strigolactone receptor, DWARF14 (D14), was identified in all plants except the plant parasite species *Striga* (*Striga hermonthica*) (Toh et al., 2015). This receptor recognizes all diverse strigolactone structures (Yoneyama and Brewer, 2021). For the first time, D14 was described as a component of the strigolactone signaling pathway in 2009, based on the analysis of semidwarf and highly branched rice (*Oryza sativa*) mutant (Arite et al., 2009). Mutant *osd14* was insensitive to GR24 (synthetic strigolactone analog) treatment and exhibited higher endogenous levels of 2^{0}-epi-5-deoxystrigol, when compared to wild-type plant (cultivar Shiokari), similar to other known strigolactone signaling rice mutants (Ishikawa et al., 2005). Positional cloning revealed that mutation in the gene encoding a/b-hydrolase superfamily protein is responsible for *osd14* phenotype (Arite et al., 2009). In the following years, D14 orthologs were identified in a wide range of plant species, including SL-induced parasitic plants (Guercio et al., 2023), uncovering the role of D14 in strigolactone perception. First, studies on *Arabidopsis* allowed describing the specificity of D14 to strigolactone signaling pathway by exclusion its role in the signaling cascade of karrikins (Waters et al., 2012), the plant growth regulators with a structure similar to strigolactones (both share a substituted butenolide moiety) (De Cuyper et al., 2017). At the same time, work on another model plant in strigolactone studies, petunia (*Petunia hybrida*), allowed identification of the D14 ortholog DAD2 (decreased apical dominance2) as strigolactone receptor. This conclusion was drawn based on the observation of decreased melting temperature of DAD2 in the presence of GR24 (Hamiaux et al., 2012), which indicated that binding strigolactone molecule by receptor results in a conformational change of the latter. Moreover, in those studies, X-ray crystallography was used to solve the structure of DAD2 for the first time. Strigolactone receptor comprises a 7-stranded b sheet 'core' domain, with the canonical catalytic triad formed by Ser96, His246, and Asp217 (binding pocket),

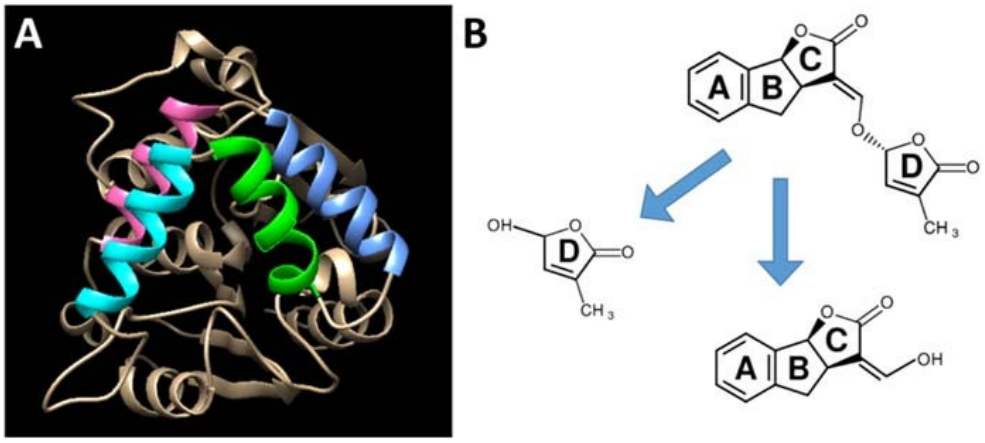

FIGURE 4.2

Strigolactone perception. (A) Four superficial alpha-helices (marked with color) form the 'helical cap' surrounding entry to the active site pocket, where the strigolactone molecule is bound and hydrolyzed. (B) Strigolactone receptor cleavages the strigolactones into the tricyclic lactone moiety (the 'ABC rings') and a butenolide moiety ('D-ring').

flanked by seven a helices (Fig. 4.2). The enzymatic activity of DAD2 was observed when the protein was mixed with GR24 (at a 1:20 M ratio), which resulted in GR24 hydrolysis. The importance of catalytic triad in strigolactone cleavage was confirmed by analysis of two versions of DAD2 with a mutation in the active sites (DAD2S96A and DAD2H246A), which lost hydrolysis activity (Hamiaux et al., 2012). Based on obtained results, the authors concluded that the hydrolytic activity of DAD2 is to produce bioactive products from a strigolactone precursor. On the other hand, the receptor can play a dual role: in signal perception and ligand degradation. However, due to the long hydrolysis time (w50% loss in 3 h), the role of the receptor in regulating strigolactone concentration was less likely (Hamiaux et al., 2012). It was proposed that AtD14 could cleavage strigolactones into the tricyclic lactone moiety (the 'ABC rings') with a butenolide moiety (the 'D-ring') (Scaffidi et al., 2012) (Fig. 4.2). Because both products of this reaction are not biologically active (Akiyama et al., 2010), it seems that one or both of them may induce the signal cascade via, i.e., conformational change of strigolactone receptor (Scaffidi et al., 2012). Further crystallographic studies confirmed that the 'D-ring' (5-hydroxy-3-methylbutenolide) is trapped in the binding pocket of D14 and changes the conformational state of the receptor. This conformational change is required for interaction with other components of the strigolactone signaling pathway (Nakamura et al., 2013). Thus, the strigolactone receptor can occur in two states: 'open' when entry to the binding pocket, which contains a catalytic triad, is available for the ligand, and 'closed' when the active pocket is occupied by the 'D-ring' and the receptor cannot bind another molecule (Kagiyama et al., 2013). ABC rings are not required for strigolactone activity (Fukui et al., 2011), and 'D-ring' subject to direct nucleophilic attack of D14

plays a key role in the strigolactone signal transduction (Kagiyama et al., 2013; Scaffidi et al., 2012). The binding pocket of D14/DAD2 is partially covered by a cap formed by four helicases Fig. 4.2 (Kagiyama et al., 2013; Nakamura et al., 2013). The importance of the entry aperture to the receptor binding pocket in strigolactone perception was demonstrated by analyzing the barley (*Hordeum vulgare*) mutant *hvd14.d*. Due to the *hvd14.d* mutation, located in one of the helicases, the diameter of D14 entry was reduced, resulting in strigolactone insensitivity (Marzec, 2016). It was also proved that the binding pocket size affects the receptor selectivity and sensitivity. When comparing two strigolactone receptors of *Striga*, ShHTL1 (hyposensitive to light1) and ShHTL7, the first was less perceptive to synthetic strigolactones due to the smaller binding pocket, compared to ShHTL7 (Toh et al., 2015; Xu et al., 2018).

Until now, all identified functional strigolactone receptors possess a highly conserved catalytic triad (Ser96, His246, and Asp217) involved in the hydrolysis of strigolactone molecules. Moreover, replacing those amino acids resulted in decreased activity and sensitivity of D14 (Hamiaux et al., 2012), which indicated that perception of the strigolactone signal is based on the degradation of the strigolactone molecule. Thus, the strigolactone receptor acts as a single-turnover enzyme that generates a ligand (the 'D ring') that remains irreversibly bound to D14/DAD2. Crystallographic studies revealed that a key role in strigolactone hydrolysis is a nucleophilic attack of a catalytic serine at the $C5^0$ position of the D ring. Modeling studies revealed the different intermediate states of connection between the receptor and D ring, indicating the dynamic of interaction between ligand and receptor (reviewed by Guercio et al., 2023). Interestingly, further studies uncover that strigolactone hydrolysis is not required to change the conformation state of the receptor. It was proved that the *Arabidopsis* mutantsedeprived enzymatic activity of strigolactone receptor ($AtD14^{S97C}$ and $AtD14^{D218A}$) can rescue (partially or entirely, respectively) the phenotype of *atd14* in an SL-dependent manner (Seto et al., 2019). Furthermore, in the mixture of AtD14 and GR24 (1:6 M ratio), all strigolactone molecules were consumed in 4 h. When a new portion of strigolactone was added, the AtD14 could still hydrolyze GR24 (Seto et al., 2019). All these data together indicate that D14 is a dual-functional receptor responsible for both the perception and deactivation of bioactive SLs. However, the perception of the strigolactone signal does not require hydrolysis since binding the strigolactone molecule already changes the conformational state of the receptor, which is necessary to signal transduction. Currently, there are still discussions about the mechanisms of strigolactone perception, and further studies are needed to obtain a complete understanding of this mechanism.

3. Core components of strigolactone signal transduction

All major components of the strigolactone signal transduction pathway were already described in many agricultural and model species in plant genetics, including *Arabidopsis* and rice (Marzec, 2016). The most critical protein

3. Core components of strigolactone signal transduction

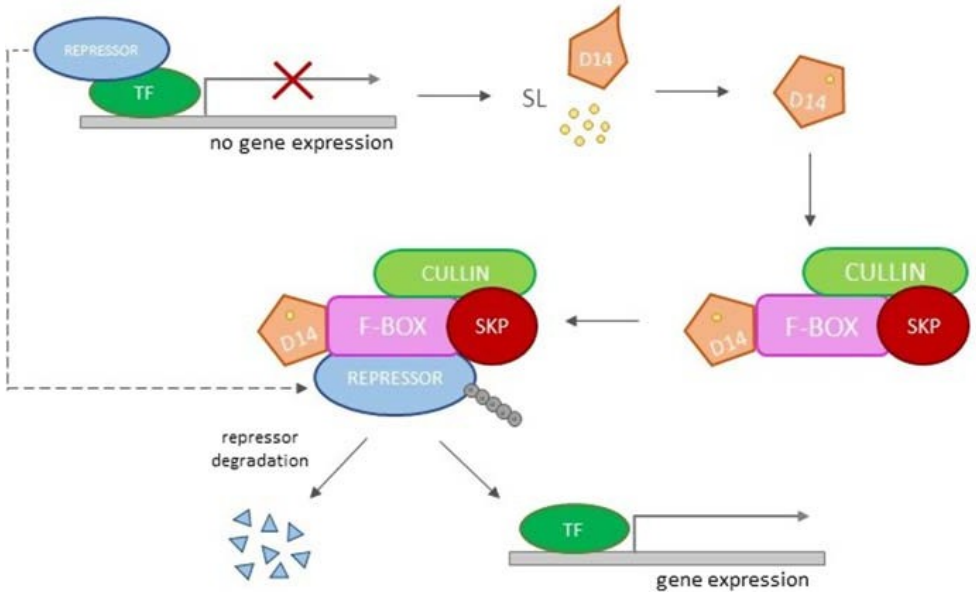

FIGURE 4.3

Scheme of strigolactone transduction pathway. In the absence of strigolactone, the expression of strigolactone inducible gene is blocked by repressor. After the strigolactone molecules are recognized and perceived by the receptor, the D14 changes its conformation to be able to interact with the F-box protein from the SKP1-cullin-F-box complex (SCF). Following, the repressor protein is marked by ubiquitination for the degradation by 26S proteasome, which releases the TF and allows the transcription of strigolactone-responsive gene.
D14, DWARF14; *TF*, transcription factor.

determining the specificity of strigolactone transduction is an F-box protein that interacts with D14 after perception of strigolactone molecule (Fig. 4.3). As an F-box protein in *Arabidopsis*, more axillary growth 2 (MAX2) confers substrate specificity to the AtCULLIN1 and *Arabidopsis* serine/threonine kinase1 (ASK1) that function together as an SCF complex, a class of E3 ligase complex that ubiquitinate target proteins to mark them for proteolysis by the 26S proteasome. Analogically, in rice, the DWARF 3 acts as an F-box protein and forms the SCF complex with OsCULLIN1 and *O. sativa* SKP1-LIKE1/5/20 (OSK1/5/20). The two-hybrid assay proved experimentally that *Petunia* DAD2 and MAX2 proteins interact in a manner dependent on strigolactone concentration (Hamiaux et al., 2012). This effect was also observed under in vivo conditions using bimolecular fluorescence complementation (BiFC) analysis in rice protoplasts (Zhao et al., 2014). The OsD14 and OsD3 dimer was assembled after GR24 treatment within the nucleus. The biochemical and crystallographic data showed that the interaction between D14 and MAX2/D3 occur through a motif of D14 only available after the conformational shift from ‘open’ to ‘closed’ state of strigolactone receptor (Yao

et al., 2016). Moreover, the binding of D14 with leucine-rich repeat domain of MAX2/D3 stabilizes the structure of D14 after its conformational change (Machin et al., 2020). This hypothesis is supported by the fact that after the breakdown of the core strigolactone signaling complex, D14 is destabilized (Zhao et al., 2015). In rice, after rac-GR24 application, the D14 was rapidly polyubiquitinated and degraded, which was impaired during analogous analyzes in *d3* mutants (Hu et al., 2017). Similarly, the proteasome-dependent degradation of D14 was induced in *Arabidopsis* by rac-GR24 treatment (Chevalier et al., 2014).

The plants harboring the mutation in the *AtMAX2/OsD3* gene exhibit the phenotype typical for strigolactone-related mutants, including semidwarf height, more shoot branches and lateral roots, abused flavonoid profile, as well as inhibited secondary growth (Ishikawa et al., 2005; Richmond et al., 2022; Stirnberg et al., 2002). Moreover, the phenotype of *max2* and *d3* plants cannot be rescued by GR24 application. Many studies have been devoted to the role of MAX2/D3 during analyzes of plant architecture or in various environmental conditions revealing the role of strigolactones in response to biotic and abiotic stresses. However, difficulties in the study of strigolactone signaling are compounded by the existence of the karrikin, because MAX2/D3 is a common point in strigolactone and karrikin signaling pathways (Nelson et al., 2011). Thus, the analyses carried out on mutants in the genes encoding the F-box protein from the SCF complex (*AtMAX2*/*OsD3*) in terms of the functioning of the strigolactones signaling pathway seem controversial. *max2* mutants were shown to be insensitive to both strigolactone and karrikin treatment and show phenotypes that can be attributed to both, while receptor mutants: *d14 and kai2 are* specifically insensitive to strigolactone and karrikin, respectively (Smith and Li, 2014; Swarbreck et al., 2020). Since not all strigolactone signaling transduction pathway components are strigolactone-specific, it was postulated that mutants in the D14 should be exclusively included in studies disclosing the role of strigolactones in plants (Waters et al., 2012).

Regarding proteolytic targets of the D14-SCF$^{MAX2/D3}$ complex, DWARF53 (D53) and three suppressor of max2-like (SMXL) family members have been identified in rice and *Arabidopsis*, respectively (Jiang et al., 2013; Soundappan et al., 2015; Wang et al., 2015; Zhou et al., 2013). Screening SL-insensitive rice mutants presenting semidwarf phenotypes and an exaggerated number of tillers compared to WT plants allows for identifying D53 as involved in SL signaling (Zhou et al., 2013). Overexpression of *D53* gene both in *d53* and WT plants resulted in increased branching, suggesting that mutation in D53 (amino acid substitutiondR812T, and deletion of five amino acidsdGKTGI$_{817}$) confers a gain of function (Zhou et al., 2013). Similarly, the participation of SMXL6/7/8 in shoot tillering in *Arabidopsis* was confirmed (Soundappan et al., 2015; Wang et al., 2015). The loss of function of redundant *SMXL6,7,8* suppresses the typical strigolactone-related phenotype of *d14* or *max2* mutants. Moreover, deleting the conserved 'FRGKT' motif in SMXL7 prevents this protein from strigolactone-responsive degradation, similar to rice D53, indicating that SMXL6/7/8 acts as D53 homologs (Soundappan et al., 2015). Both SMXL6,7,8 and D53 possess a conserved ethylene-responsive element binding factor-associated

amphiphilic repression motif, which is essential for interaction with topless or topless-related to induce their oligomerization to form a repressorecorepressorenucleosome complex (Jiang et al., 2013; Mach, 2015). It was experimentally shown that D53 and SMXL6,7,8 interact with TRP proteins to repress strigolactone-related transcription (Wang et al., 2015). More importantly, the SMXL7 and D53 showed a rapid degradation in D14-MAX2/D3 and proteasome-dependent manned after the strigolactone treatment of wild-type plants (Liang et al., 2016; Wang et al., 2015; Zhou et al., 2013). Furthermore, both in vitro and *in planta* interactions have been noted between all three core strigolactone signaling proteins in *Arabidopsis* and rice forming D14-MAX2-SMLX7 and D14-D3-D53 complexes, indisputably confirming the contribution of each protein to strigolactone signaling (Liang et al., 2016; Wang et al., 2015; Zhao et al., 2014; Zhou et al., 2013).

In *Arabidopsis*, the SMXL family consists of eight members, where members subclade 4 (SMXL6, SMXL7, and SMXL8) are involved in strigolactone-mediated transcription regulation. In contrast, suppressor of max2 1 (SMAX1), SMAX2, and other SMXL (SMXL3, SMXL4, and SMXL5) play a role in karrikin signaling (Soundappan et al., 2015; Wallner et al., 2017). For many years, the model of strigolactone and karrikin signaling complexes has been believed to act separately. However, this idea has been challenged based on data from *Arabidopsis* hypocotyl elongation studies (Wang et al., 2020b). It was shown that strigolactone treatment enhanced the assembling of both D14-SMXL2 and KAI2-SMAX1/SMAX2 complexes. It should be noted that authors used specific molecules perceived by the D14 and KAI2 receptors, $GR24^{4DO}$ and $GR24ent^{-5DS}$, respectively. In both cases, degradation of SMAX2 repressor was observed, which raises the question about the previously postulated strigolactone/karrikins specificity of SMXL/SMAX proteins. It cannot be excluded that further years will bring the discoveries of more proteins that can function as targets for D14-$SFC^{MAX2/D3}$ complex.

4. Functional characterization of strigolactone-responsive transcription factors and downstream genes

General knowledge of the strigolactone signaling pathway and individual proteins involved in signal transduction appears to be well understood in model species. However, we still have rudimentary information about the downstream strigolactone genes, in particular, the TFs that regulate the plant's response to strigolactone. The first downstream targets of strigolactone repressor were proteins belonging to the teosinte branched1/cycloidea/proliferating cell factor1 family (Table 4.1). They all possess a so-called TCP domain, a 59 amino acid helix-loop-helix motif, that allows DNA binding and proteineprotein interactions (Wang et al., 2019). The representatives of strigolactone-responsive TCP TFs have been found in several plant species including *Arabidopsis*, rice, pea (*Pisum sativum*), wheat (*Triticum aestivum*), grapevine (*Vitis vinifera*), and maize (*Zea mays*). The branched1 (BRC1) protein and its homologs in other species are so far best-characterized strigolactone-responsive

Table 4.1 Lists of BRC1 homologs in different species, whose strigolactone-dependent expression was experimentally confirmed.

Species	BRC1 homolog	Reference
Arabidopsis thaliana	BRANCHED1, AtBRC1	Aguilar-Martı´nez et al. (2007)
Oryza sativa	FINE CULM1, OsFC1 TEOSINE BRANCHED1, OsTB1	Song et al. (2017)
Pisum sativum	BRANCHED1, PsBRC1	Braun et al. (2012)
Triticum aestivum	TEOSINE BRANCHED1, TaTB1	Liu et al. (2017)
Vitis vinifera	BRANCHED1, VvBRC1	Min et al. (2021)
Zea mays	TEOSINE BRANCHED1, ZmTB1	Guan et al. (2012)

TFs. The BRC1 is well known to act locally in buds and regulates the shoot branching by inhibiting the axillary bud outgrowth.

A role for BRC1 as a downstream strigolactone component was first reported in *Arabidopsis* (Aguilar-Mart´ınez et al., 2007) and pea (Braun et al., 2012). *AtBRC1* expression was upregulated by strigolactone application, and *atbrc1* mutants displaying highly shoot branching phenotype could synthesize strigolactone, but did not respond to strigolactone treatment. Both strigolactone-insensitive (*atmax2*) and strigolactone-depleted (*atmax1,3,4*) plants showed significantly reduced accumulation of *AtBRC1* transcripts (Aguilar-Mart´ınez et al., 2007). Similarly, potent inhibition of axillary bud outgrowth was observed in wild-type plants and *psrms1* mutants 10 days after GR24 treatment, in contrast to *psbrc1* where continuation of branching was still observed (Braun et al., 2012). Undisputed evidence for regulating *BRC1* activity by strigolactone is its constitutive upregulation in mutants deficient in SMXL6/7/8 proteins (Seale et al., 2017; Soundappan et al., 2015; Wang et al., 2015). However, there is no experimental confirmation that BRC1/FC1/TB1 protein is the direct target of strigolactone repressor, but it is a prominent candidate for further studies that aim the identification of other TFs and downstream strigolactone-responsive genes. The research conducted on wheat shed new light on this issue. To date, several studies link the function of miR156 and its targets belonging to squamosa promoter binding protein-like (SPL) family with the strigolactone-mediated shaping of plant architecture (Chen et al., 2015; Luo et al., 2012). Indeed, in the transgenic high-tillering line overexpressing the *TaMIRNA156* gene, the expression of *TaTB1* was significantly reduced (Liu et al., 2017). The authors demonstrate that the strigolactone signaling repressor TaD53 can directly interact with N-terminal domain of mir156-controlled SPL3/17. Most importantly, using the transient expression system in tobacco (*Nicotiana benthamiana*), the TaSPL3/17-mediated transcriptional activation of *TaTB1* can be largely repressed by TaD53 (Liu et al., 2017). Thus, it seems that the shaping of plants' shoot architecture induced by strigolactone might be regulated through D14-D53-SPL3/17-

4. strigolactone-responsive transcription factors and downstream genes

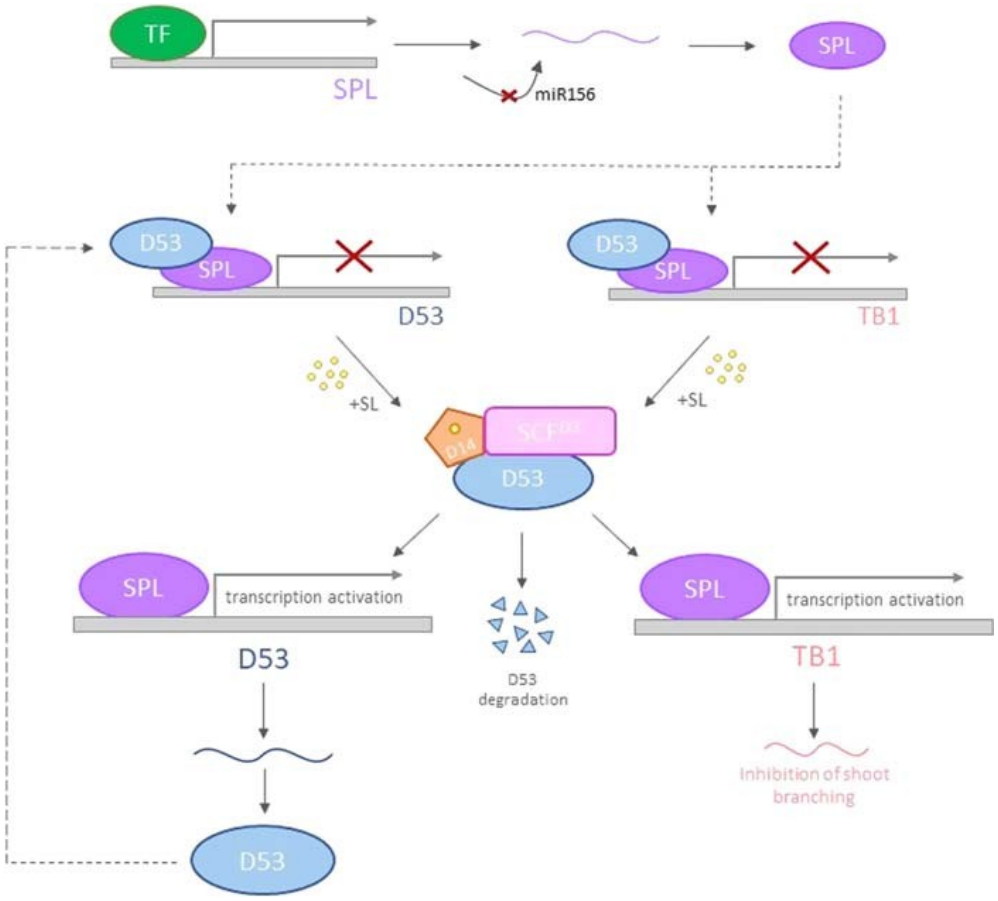

FIGURE 4.4

Strigolactone-mediated regulation of shoot branching based on wheat and rice studies. The expression of *TB1* gene is directly regulated by protein from SPL family. In the presence of strigolactone, D53 is targeted by SCF^{D3} complex for degradation, resulting in the expression of *TB1* to promote inhibition of shoot branching. On the other hand, the *D53* gene undergoes regulation by SPL, thus forming a feedback response of SL-induced D53 expression.
D, DWARF; SCF^{D3}, SKP-cullin-D3 complex; *SPL*, squamosa promoter binding protein-like; *TB1*, teosine branched1; *TF*, transcription factor.

TB1 pathway (Fig. 4.4). Similar conclusions were reached during studies conducted on rice, where direct interaction between OsD53 and OsSPL14, also called as ideal plant architecture 1 (IPA1) has been experimentally confirmed both under in vitro conditions by yeast two-hybrid assay, as well as under in vivo condition by BiFC (Song et al., 2017). Further studies showed that IPA1 directly binds to the negative regulator of tiller bud outgrowth promoter, *OsTB1*, to suppress rice tillering. However, strigolactone treatment does not affect the mRNA or protein accumulation of

IPA1. Furthermore, it turned out that IPA1 can directly bind to the *OsD53* promoter and play a critical role in the feedback regulation of SL-induced D53 expression (Fig. 4.4). These findings provide new insights into understanding the strigolactone repressor's function and the strigolactone signaling network. Inconsistent results regarding BRC1/FC1/TB1 in plant branching regulation have been obtained in some monocotyledonous species. Although the high-tillering phenotypes of single (*Ostb1*) or double (*Ostb1/Osd17*) rice mutants were not rescued after the GR24 treatment, the *OsTB1* expression was not significantly affected by GR24 in wild-type (Minakuchi et al., 2010). Additionally, the overexpression of *OsTB1* only partially rescues the branching phenotype of *osd3* mutant, suggesting that rice may involve an alternative pathway leading to the inhibition of axillary bud outgrowth. In line with these observations were results obtained during the analysis of maize, where the expression of *ZmTB1* gene was not reduced in *Zmccd8* mutant or upregulated by GR24 treatment (Guan et al., 2012).

Besides regulating plant shoot branching, strigolactones also participate in secondary growth by promoting cambium cell proliferation (Wani et al., 2021). During secondary growth, cambium cells can multiply to maintain the meristematic cell population or differentiate into xylem or phloem. The balance between these two opposite development poles is strictly controlled by many environmental and endogenous factors (Wang, 2020). Based on genetic and biochemical analyses of *Arabidopsis*, it was demonstrated that BRI1-EMS-suppressor1 (BES1) plays a key role in inhibiting cambium activity by suppressing the expression of WUSCHEL-related homeobox4 (WOX4) in a strigolactone-mediated way (Fig. 4.5) (Hu et al., 2022). As it turned out, the *BES1* RNA interference (*BES1*-RNAi) line presented a phenotype typical for the strigolactone-depleted or strigolactone-insensitive mutants. Additionally, the *WOX4* transcripts level was significantly higher in transgenic plants with knock-down of *BES1* and lower in *atmax3* and *atmax2* mutants, when compared to wild-type. Further, both chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) and luciferase reporter system in tobacco showed that BES1 could directly bind to the promoter of *WOX4* and inhibit its expression (Hu et al., 2022). Since BES1 has been identified as a direct target of SFC^{MAX2} complex in a strigolactone-dependent manner, the authors proposed that BES1 acts as a repressive TF and coregulator of SMLX proteins (Hu et al., 2020; Wang et al., 2013). Additionally, the interaction between SMXL proteins and BES1 was proved experimentally in *in vivo* conditions, thus suggesting that SMLXs-BES1 complex is degraded by D14-SCF^{MAX2} after strigolactone perception (Hu et al., 2020, 2022). All these findings suggested that BES1 inhibits the proliferation of vascular cambium cells by repressing *WOX4* expression, known for its role in regulating the secondary growth of plants (Fig. 4.5).

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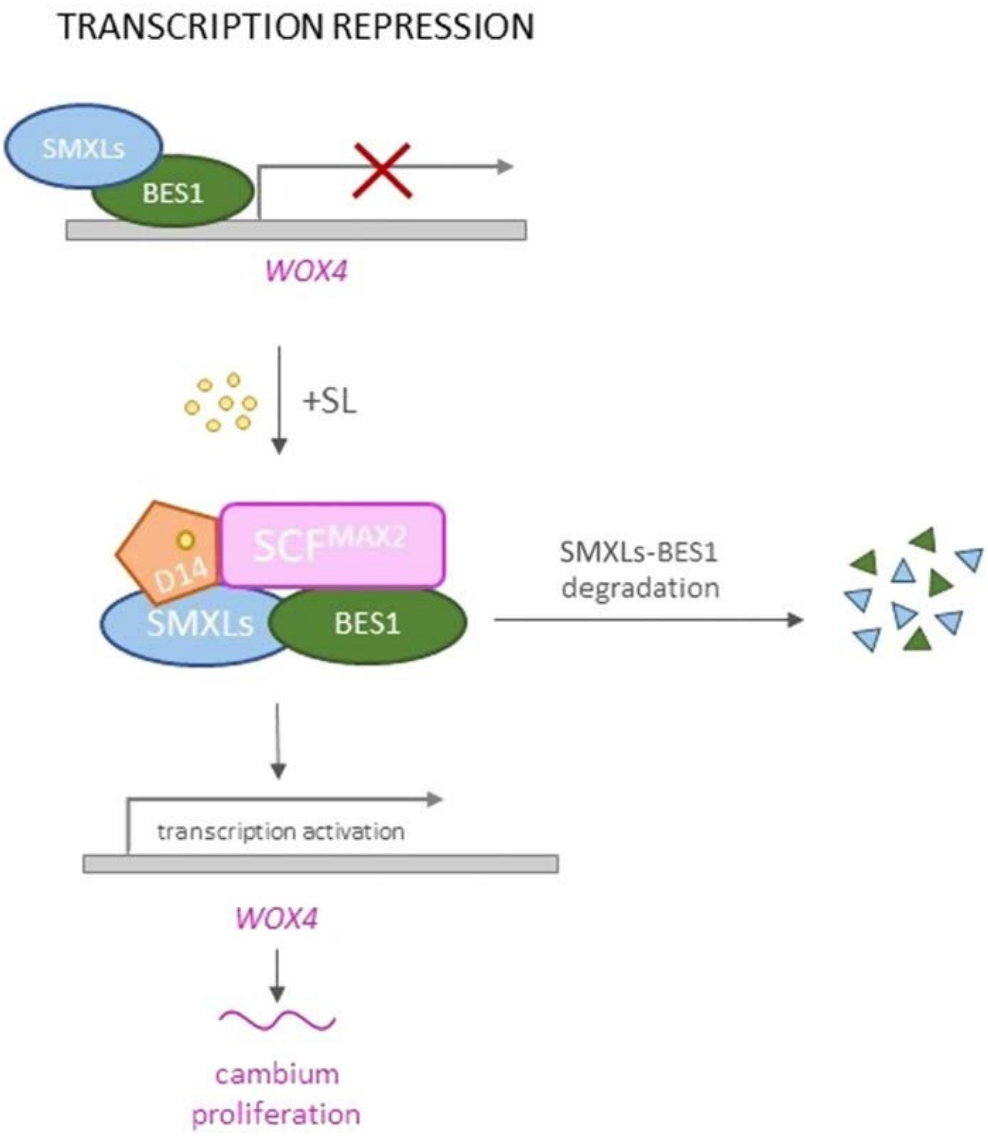

FIGURE 4.5

Strigolactone-mediated regulation of vascular cambium cells activity in *Arabidopsis*. The expression of *WOX4* gene is directly inhibited by BES1, acting as a coregulator of SMXL proteins. In the presence of SL, both SMXLs and BES1 are targeted by SCF^{MAX2} complex for degradation, resulting in the expression of *WOX4* to promote cambium proliferation. *BES1*, BRI1-EMS-suppressor1; *D14*, DWARF14; SCF^{MAX2}, SKP-cullin-MAX2 complex; *SMXL*, suppressor of MAX2-like; *WOX4*, WUSCHEL-related homeobox4.

For a very long time, the limited knowledge of strigolactone-responsive genes has seriously hampered our understanding of strigolactone signal transduction. Previous studies have focused only on the functional analysis of single genes suspected of regulating plant response to strigolactone. Recently, Wang and colleagues used synthetic strigolactone ($GR24^{4DO}$) to identify 401 strigolactone-responsive genes in *Arabidopsis*, 90% of which were not previously reported (Wang et al., 2020a). The bioinformatics analysis of this gene set revealed complex cross-talk between strigolactones and abscisic acid, auxin, as well as karrikins. Moreover, the role of strigolactones in microtubule function, drought resistance, and biosynthesis of carotenoids or flavonoids was postulated based on the gene ontology analyses (Wang et al., 2020a). Further, the transcriptomic analysis showed that exogenous application of strigolactone might activate or repress 24 and 14 genes encoding TFs, respectively. The SL-dependent responsiveness was experimentally confirmed in three of themd*BRANCHED1* (*BRC1*), *TCP domain protein1* (*TCP1*), and *production of anthocyanin pigment1* (*PAP1*), which are involved in the control of shoot branching, leaf shape, and anthocyanin biosynthesis, respectively (Fig. 4.6). The *atbrc1* mutant could completely suppress the *Arabidopsis* triple strigolactone repressor *smxl6,7,8* mutant, as well as the overexpression of *AtTCP1* resulted in more rounded leaves in both wild-type and triple *smxl6,7,8* mutant (Wang et al., 2020a). Similarly, the anthocyanin biosynthesis was normalized in *smxl6,7,8* after a knock-out of *AtPAP1* gene. The most shift in the current view of transcriptional repressors in phytohormone signaling was due to identifying high-quality targets of AtSMXL6. The ChIP-seq assays proved that AtSMXL6 might bind to genome regions of 729 genes, including the promoter sequences of *SMXL6,7,8,* which was additionally confirmed by electrophoretic mobility shift assays (Wang et al., 2020a). The fact that SMXL6 acts as an autoregulating TF and represses the expression of other SMXL genes (Wang et al., 2020a) is an unexpected twist of our understating of repressors in plant hormone signaling pathways involving SCF-mediated degradation (Tang and Chu, 2020). To maintain the homeostasis of strigolactone signaling, the SMXL6 represses the signal transduction by both proteineprotein interactions with specific TFs, but also by directly targeting the DNA and regulating the transcription of genes in higher plants (Fig. 4.6). The transcriptional studies were also conducted on grapevine plants, where young cuttings were subjected to decapitation and treatment with rac-GR24 (Min et al., 2021). The results showed that grapevine bud growth was significantly induced in the control, decapitated plants group, but largely inhibited after the rac-GR24 application. The differential gene expression analysis reveals 1390 genes encoding TFs, including members of MYB, HLH, WRKY, HSP70, bZIP, TCP, and GRAS family, some of which are known for plants architecture regulation (Finlayson, 2007; Li et al., 2003; Schmitz and Theres, 2005; Yao et al., 2022).

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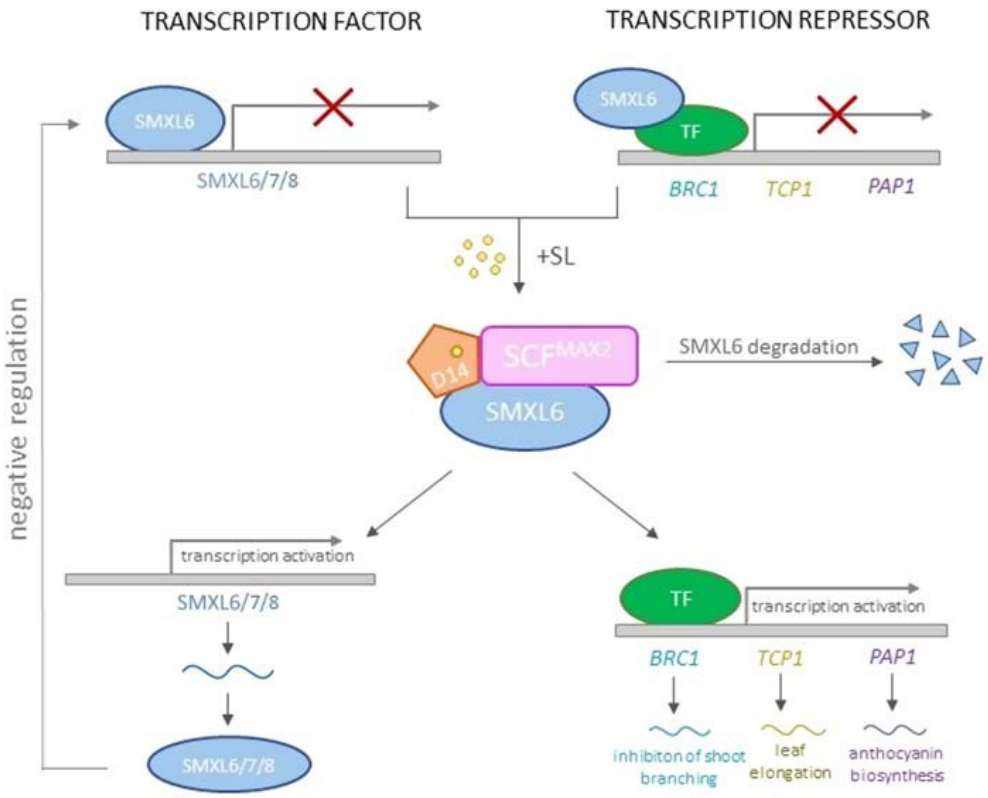

FIGURE 4.6

Working model of SMXL6-mediated regulation of strigolactone-responsive genes in *Arabidopsis*. In the absence of strigolactones (SLs), SMXL directly binds to the transcription factor (TF), which recognizes and targets the promoter sequence of *BRC1*, *TCP1*, and *PAP1* genes. The interaction between SMXL and TF represses the expression of SL-responsive genes. Meanwhile, SMXL6 binds directly to the promoters of *SMXL6,7,8*, functioning as an auto/regulating repressive TF. As soon as the SL molecules are present, the SFC^{MAX2} complex is assembled and triggers the ubiquitin-mediated degradation of SMXL6, thus releasing the transcriptional repression. In parallel, the degradation of SMXL6 enables the transcription of the remaining SMXL genes to be activated. Newly synthesized SMXL6, involved in the negative feedback loop, inhibits the transcription of *SMXLs* genes.

BRC1, branched1; *D14*, DWARF14; *PAP1*, production of anthocyanin pigment1; SCF^{MAX2}, SKP-cullin-MAX2 complex; *SMXL*, suppressor of MAX2-like; *TCP1*, TCP domain protein1; *TF*, transcription factor.

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ROZDZIAŁ III.3

Strigolactone insensitivity affects differential shoot and root transcriptome in barley

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PLANT GENETICS • ORIGINAL PAPER

Strigolactone insensitivity affects differential shoot and root transcriptome in barley

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Abstract

Strigolactones (SLs) are plant hormones that play a crucial role in regulating various aspects of plant architecture, such as shoot and root branching. However, the knowledge of SL-responsive genes and transcription factors (TFs) that control the shaping of plant architecture remains elusive. Here, transcriptomic analysis was conducted using the SL-insensitive barley mutant *hvd14.d* (carried mutation in SL receptor DWARF14, HvD14) and its wild-type (WT) to unravel the differences in gene expression separately in root and shoot tissues. This approach enabled us to select more than six thousand SL-dependent genes that were exclusive to each studied organ or not tissue-specific. The data obtained, along with *in silico* analyses, found several TFs that exhibited changed expression between the analyzed genotypes and that recognized binding sites in promoters of other identified differentially expressed genes (DEGs). In total, 28 TFs that recognize motifs over-represented in DEG promoters were identified. Moreover, nearly half of the identified TFs were connected in a single network of known and predicted interactions, highlighting the complexity and multidimensionality of SL-related signalling in barley. Finally, the SL control on the expression of one of the identified TFs in HvD14- and dose-dependent manners was proved. Obtained results bring us closer to understanding the signalling pathways regulating SL-dependent plant development.

Keywords Barley · DWARF14 · *Hordeum vulgare* · Root · Shoot · Strigolactones · Transcriptome

Introduction

Strigolactones (SLs) are phytohormones involved in the control of plant architecture, including shoot branching, plant height (Gomez-Roldan et al. 2008; Umehara et al. 2008) as well as root elongation and branching (Koltai 2011). Grafting studies revealed that SLs may be synthesized in roots (Beveridge 2000; Booker et al. 2005) and transported to the aboveground organs via SL-specific transporters (Kretzschmar et al. 2012). On the other hand, SLs are also secreted via roots to the rhizosphere, where they act as signal molecules in communication with other organisms, such as bacteria, fungi and other plants (Kee et al. 2023). Moreover, studies from recent years indicate that SLs play critical functions in the plant response to stresses, especially abiotic ones (Yoneyama et al. 2012). Plants adapt to changing environmental conditions via SL-mediated modulation of underground and aboveground organ development (Trasoletti et al. 2022). Under control conditions, SLs inhibit the shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008). Thus, mutants deficient in SL biosynthesis or signalling exhibit a bushy phenotype. In contrast, the application of SLs reduces shoot branching (reviewed by Kelly et al. 2023). The SL receptor D14 (DWARF14) recognizes the SL molecule, which changes the receptor conformation to facilitate SL signalling complex assembly (Marzec and Brewer 2019). This complex binds the SL repressor D53 (DWARF53), which undergoes proteasomal degradation in an SL-dependent manner (Zhou et al. 2013) to activate SL-dependent transcription factors (TFs) and their target genes. The key SL-dependent TF involved in the regulation of shoot branching is BRC1 (BRANCHED1). Initially, BRC1 was identified in *Arabidopsis* (*Arabidopsis thaliana)*

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(Aguilar-Martínez et al. 2007), and its ortholog TB1 (TEOSINTE BRANCHED1) was characterized in monocotyledons rice (*Oryza sativa*) (Takeda et al. 2003) and maize (*Zea mays*) (Doebley et al. 1997). It was shown that *BRC1/TB1* expression is limited to the axillary buds, which negatively correlates with bud outgrowth (Takeda et al. 2003; Aguilar-Martínez et al. 2007). The bushy phenotype of *brc1* mutants in *Arabidopsis* and pea (*Pisum sativum*) cannot be reversed by SL treatment, indicating the BRC1 action downstream of SLs (Brewer et al. 2009). Moreover, exogenous SLs elevate the expression of *BRC1* in wild-type (WT) plants, while *BRC1* expression is reduced in SL mutants (Dun et al. 2012), clearly showing that SLs regulate shoot branching via BRC1/TB1. On the other hand, the outgrowth of axillary buds depends on the export of auxin from buds via PIN-FORMED (PIN) protein efflux carrier proteins (Wiśniewska et al. 2006). It was shown that SL biosynthesis mutants exhibited increased PIN1 abundance and therefore increased auxin transport, which results in the highly branched phenotype (Bennett et al. 2006). At the same time, SL treatment disturbs the cellular localization of PIN1 (Shinohara et al. 2013). Thus, SLs control shoot architecture via modulation of BRC1/TB1 activity and control of auxin transport. To date, the role of SLs in inhibiting axillary bud outgrowth was the best characterized function of SLs in plants. Moreover, the results obtained for different species are consistent and reproducible (Kelly et al. 2023).

The role of SLs in shaping root architecture was proposed in 2011 based on studies in *Arabidopsis* (Kapulnik et al. 2011; Ruyter-Spira et al. 2011). Both SL biosynthesis and signalling mutants developed a higher number of lateral roots compared to the WT, and SL treatment reduced the lateral root number in WT and SL biosynthesis mutants, but not in SL signalling mutant (Kapulnik et al. 2011; Ruyter-Spira et al. 2011). The inhibitory SL effect on lateral root density was also observed in other species, such as *Lotus japonicus* (Liu et al. 2013), *Medicago truncatula* (De Cuyper et al. 2015) and barley (*Hordeum vulgare*) (Marzec et al. 2016). SL biosynthesis and signalling mutants in rice developed a similar number of lateral roots to the WT (Arite et al. 2012); the SL treatment still reduced lateral root density in WT rice plants (Sun et al. 2014). Reduction of lateral root density in rice after SL application was linked with decreased expression of several genes encoding PINs and inhibition of auxin transport from shoot to root (Arite et al. 2012; Sun et al. 2014). Moreover, an elevated auxin concentration was observed in the root tissue of rice SL biosynthesis mutant (*Osd10/17*) (Sun et al. 2014, 2015). When the standard concentration of auxin is present in the *Arabidopsis* root, SLs regulate the cellular localization of PINs and thus repress the expression of auxin-dependent genes and reduce the lateral root number (Ruyter-Spira et al. 2011; Zhang et al. 2020a, b). Conversely, increased auxin concentration in root SLs promotes the development of lateral roots (Ruyter-Spira et al. 2011; Mayzlish-Gati et al. 2012). In response to various stresses, such as nutrient deficiency, drought, salinity, or increased heavy metal concentration, the impact of SLs on root system development became more enigmatic (Marzec and Melzer 2018; Sun et al. 2022). Hence, the role of SLs in root development is much more complicated than in the case of shoots, and it is also affected by many factors, such as growing conditions or plant age.

In recent years, significant progress in understanding the function of SLs has been made thanks to high-throughput comparative analyses of SL mutants or SL-treated vs. untreated plants. Wang and co-workers identified 401 SL-dependent genes in *Arabidopsis*, including three TFs involved in SL signal transduction. Besides well-known BRC1, the TFs which control anthocyanin biosynthesis (PRODUCTION OF ANTHOCYANIN PIGMENT 1, PAP1) or leaf development (TCP DOMAIN PROTEIN 1, TCP1) were found to be under the control of SLs (Wang et al. 2020). Analyses of transcriptome changes mediated by SLs or auxin (indole-3-acetic acid; IAA) in tomato (*Solanum lycopersicum*) shoots revealed a higher number of differentially expressed genes (DEG) after auxin application. However, among the smaller number of genes whose expression was altered by SL treatment, the upregulated genes of the auxin signalling pathway were found, indicating the crosstalk between SLs and auxin in tomato (Zhan et al. 2018). At the same time, melon (*Cucumis melo*) root transcriptome analyses revealed the crosstalk between SLs and auxin in promoting adventitious root growth (Li et al. 2023). Root transcriptome was also investigated for rice WT and SL biosynthesis mutant in response to phosphorus starvation and SL application. Those experiments uncovered the enzyme METHYL TRANSFERASE (Os01g0700300) to be involved in SL biosynthesis (Haider et al. 2023), while treatment of apple rootstock M26 with SL synthetic analogue GR24 or SL inhibitor Tis108 revealed SLs to promote adventitious shoot formation, facilitating the identification of more than 10,000 potentially SL-responsive genes (Asghar et al. 2022). Finally, the role of SLs in plant response to drought was investigated via transcriptome analyses in various species, including *Arabidopsis* (Li et al. 2020a; Korwin Krukowski et al. 2023), rice (Yoo et al. 2017) and barley (Daszkowska-Golec et al. 2023). Based on these results, the molecular basis of the role of SL in response to drought stress was described, including interaction with abscisic acid, increased synthesis and deposition of waxes or ROS scavenging. Moreover, the first SL-dependent TFs that can mediate the adaptation of plants to water deficit have been identified.

In the presented study, we use a previously characterized barley line *hvd14.d*, which is SL-insensitive due to the mutation in SL receptor HvD14 (Marzec et al. 2016), to investigate the role of SL in the control of shoots and

roots architecture. The *hvd14.d* line has been characterized to exhibit the SL-insensitivity phenotype: semi-dwarf and highly branched shoot, as well as a root system composed of shorter seminal roots, which developed a more significant number of lateral roots, compared to the WT (Marzec et al. 2016). Moreover, *hvd14.d* mutant is more sensitive to drought (Marzec et al. 2020), which was also observed for SL-insensitive mutants in other species (Haider et al. 2018; Li et al. 2020a; Korwin Krukowski et al. 2023). Here, we use *hvd14.d* line to uncover tissue-specific SL-dependent mechanisms disturbed in this line, which affects barley shoot and root phenotype. The transcriptomic differences between *hvd14.d* and its WT were investigated separately for the shoot and root tissue. That approach allowed us to dissect the SL-related regulatory mechanisms specific to each investigated organ and those not tissue-specific.

Materials and method

Plant material, growth conditions and hormone treatment

Two genotypes were used in the described studies: wild-type variety Sebastian and *hvd14.d* mutant obtained after chemical mutagenesis (Szurman-Zubrzycka et al. 2018). Mutant *hvd14.d* is insensitive to strigolactones due to the mutation in strigolactone receptor HvD14 (Marzec et al. 2016).

For the RNAseq experiment, plants were grown in hydroponic conditions for up to 21 days. Six plants were placed in the 1.5 l container filled with ½ Hoagland solution (Hothem et al. 2003). The medium was replaced every week. Plants were placed in the greenhouse under a 20/18 °C day/night, 16/8 photoperiod and 420 µE m^{-2} s^{-1} light intensity. Total root length, lateral root length and density were determined using an Epson scanner and WINRHIZO software (Regent Instruments Inc.).

For the spraying experiments, five plants were sown in the pot (7.5 × 7.5 × 10 cm) filled with soil garden. Two-week-old seedlings were sprayed with 1 or 10 µM of $GR24^{5DS}$ (StrigoLab, Turin, Italy). Control plants were sprayed with a mock solution (0.01% acetone). Tissue for RT-qPCR analyses were collected from plants before treatment and after 0.5, 1 and 3 h after treatment.

RNA isolation and RNA sequencing

For RNA-isolation analyses, plant tissue (shoot and root) was collected in four biological replicates, each containing tissue from four seedlings. Samples were frozen immediately in liquid nitrogen; RNA was isolated using the mirVana miRNA Isolation Kit (ThermoFisher Scientific, catalogue number: AM1560). Library construction and sequencing (150-nt paired-end reads) on Illumina NovaSeq™ 6000 platforms were performed by the Novogene Genomics Service (Cambridge, United Kingdom). The Novogene Genomics Service also provided basic data analysis by applying their RNAseq pipeline. Genes with adjusted p-value < 0.05 and $\log_2$FC ≥ 1 or ≤ −1 were considered differentially expressed.

RT-qPCR

RNA was extracted as described previously, in four biological replicates, each containing tissue from five seedlings. RevertAid First Strand cDNA Synthesis Kit (Product No. K1621, Life Technologies) was used for cDNA synthesis. Diluted cDNA (1:4, cDNA:water) was used for RT-qPCR reactions performed using LightCycler FastStart DNA Master SYBR Green (Product No. 12239264001, Roche) and LightCycler 480 Instrument II (Roche). Relative expression level of HORVU.MOREX.r2.1HG0041130 (F: AGGGAC CTGGAGTGGTTCTT, R: AACACCAGCGTCTTCCTG AC) calculated and normalized to the internal control, the EF1 gene (Elongation factor 1-α; F: CCCTCCTCTTGG TCGTTTTG; R: ATGACACCAACAGCCACAGTTT). Data were analyzed using LinRegPCR (Ramakers et al. 2003). Four biological replicates were analyzed for each time point in two technical replicates. A relative expression level was presented to control, fixed as 1. Data are presented as mean ± SE of $2^{-\Delta\Delta Ct}$ in each case. Statistical analyses were performed using the t-test (*p < .05; **p < .01; ***p < .001).

Gene ontology

Gene ontology (GO) enrichment was performed using ShinyGO 0.77 tool (http://bioinformatics.sdstate.edu/go/) (Ge et al. 2020). Gene lists from Supplementary Table 1 were used as a query, and the following settings were used: FDR cutoff: 0.05, pathways to show: 20, min. pathway size: 2, max. pathway size: 2000. Treemaps for the GO biological process were prepared using the ReviGO tool (http://revigo.irb.hr/) (Supek et al. 2011). Plot size was adjusted to the Log10 p-value of the GO-term enrichment. Only the biological process GO category was used. The p-value of each GO term was obtained using the AgriGO tool (http://systemsbiology.cau.edu.cn/agriGOv2) (Tian et al. 2017).

TF prediction and promoter analysis

Amino acid sequences of all identified DEG were obtained using BioMart Ensemble Plant (http://plants.ensembl.org/info/data/biomart/index.html) v56 from 'Hordeum vulgare TRITEX genes (Morex_V2_scaf)' datasets. Those sequences were used as a query in the 'Transcription Factor Prediction' tool from PlanRegMap (http://planttfdb.gao-lab.

org/prediction.php) (Tian et al. 2019). As a result, probable TFs (with MLOC IDs) and their *Arabidopsis* orthologs were obtained (Supplementary Data 4).

Promoter sequence (1500 bp before START codon) of all identified DEG were obtained using BioMart Ensamble Plant (http://plants.ensembl.org/info/data/biomart/index.html) v56 from 'Hordeum vulgare TRITEX genes (Morex_V2_scaf)' datasets. Promoter sequences were screened using the 'Binding Site Prediction' tool from PlanRegMap (http://planttfdb.gao-lab.org/prediction.php) (Tian et al. 2019). Using the threshold p-value $\leq 1e^{-4}$, the lists of all TF binding sites in the promoter region were obtained (Supplementary Data 5).

To identify the TFs which possess significantly over-represented targets in DEG lists, previously obtained lists were analyzed with the 'TF Enrichment' tool from PlanRegMap (http://planttfdb.gao-lab.org/prediction.php) (Tian et al. 2019) using the following settings: species, *Hordeum vulgare*; method, motif; threshold p-value ≤ 0.05.

Results

Insensitivity to SLs affects shoot and root architecture in barley

Chemical mutagenesis and the TILLING strategy allowed the identification of a barley mutant with a mutation in the gene encoding the strigolactone receptor, *HvD14*. This mutation has been shown to change the conformation of the protein, narrowing the entrance to the active site, resulting in insensitivity to strigolactone. As reported previously, semi-dwarf barley mutant *hvd14.d* produces an almost two times higher number of tillers than the parent variety Sebastian (WT) when plants were grown in the soil (Marzec et al. 2016). Similar results were obtained for hydroponic conditions when comparing 3-week-old plants of both genotypes. The number of shoot branches in Sebastian (3.1 ± 0.61) was 40% lower than *hvd14.d* (5.1 ± 0.68). Additional assessment of phenotypic traits, the *hvd14.d* shoot height was 20% lower than in Sebastian (Fig. 1). The same number (seven) of seminal roots in both genotypes were observed, but the length of the longest seminal root was reduced in *hvd14.d* (65% of that noted for Sebastian). On the other hand, the total length of the root system in both genotypes was similar (984.9 ± 93.43 and 1023.3 ± 103.41 cm for Sebastian and *hvd14.d*, respectively) (Fig. 1). Those results can be explained by the more significant number of lateral roots in the mutant, which is in line with previous findings (Marzec et al. 2016). Indeed, under hydroponic conditions, 3-old-week *hvd14.d* plants exhibited a higher density of lateral roots than Sebastian. Still, the length of lateral roots was similar in both genotypes (1.5 ± 0.22 and 1.4 ± 0.25 cm for Sebastian and *hvd14.d*, respectively) (Fig. 1).

Transcriptomic differences between Sebastian and hvd14.d

Gene expression was investigated separately for the shoot and root tissues of 3-week-old plants grown in hydroponics.

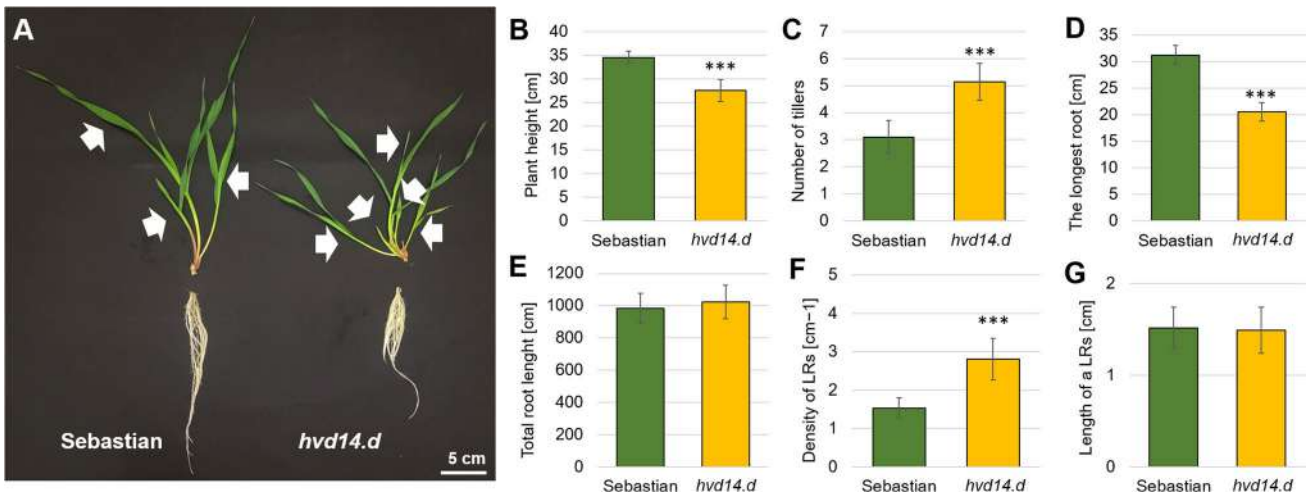


Fig. 1 The phenotype of 3-week-old seedlings of Sebastian (wild-type) and *hvd14.d* (SL-insensitive mutant). **A** Differences in root and shoot between both genotypes. **B** Mutant *hvd14.d* exhibited a semi-dwarf phenotype and **C** produced significantly higher tillers than Sebastian. **D** Despite the shorter seminal roots of *hvd14.d*, the **E** total root length of both genotypes is similar. **F** Mutant *hvd14.d* developed more lateral roots than Sebastian, but **G** the length of lateral roots in both genotypes is similar. Asterisks indicate statistically significant differences between samples in a paired Student's t-test (***correspond to p-values of $p < 0.001$; white arrows indicate tillers). LRs, lateral roots

A comparison of *hvd14.d* shoot transcriptome (d14_S) vs Sebastian shoot (Seb_S) revealed 1278 differentially expressed genes (DEG); 486 up, and 792 downregulated (adjusted p-value < 0.05 and $|\log_2(\text{FoldChange})| \geq -1$ or ≤ 1), while the comparison of root transcriptome (d14_R vs Seb_R) revealed an almost five times higher number of DEGs: 5424 (1905 up and 3519 downregulated) (Fig. 2, Supplementary Data 1). Analysis of these data revealed three sets of genes: (1) Genes differentially expressed in both shoot and root between genotypes were described as SL-related common genes (SL_C; 65 up, 157 downregulated), (2) SL-specific shoot DEGs (SL_S; 421 up, 635 downregulated) and (3) SL-specific root DEGs (SL_R; 1840 up, 3363 downregulated) when *hvd14.d* was compared to Sebastian (Fig. 2, Supplementary Data 1).

Non-organ-specific DEG analysis

Gene ontology (GO) enrichment analysis (FDR > 0.05) revealed that the SL_C upregulated set of genes is mainly related to RNA processing or metabolism (among biological process; BP) and RNA binding (among molecular function; MF), which is in agreement with the over-represented cellular component (CC) GO terms for those genes, which includes ribosome, nuclear or ribonucleoprotein complex localization. At the same time, downregulated SL_C genes were characterized as involved in photosynthesis, assimilation of photosynthetic products and response to light (BP). Consistent with these enriched BPs, enriched MF terms include chlorophyll-binding and the controlling activity of enzymes involved in sugar metabolism. In contrast, enriched CC terms include plastid, plastid membrane and photosystem localizations (Supplementary Data 2). The upper-hierarchy GO-terms revealed that DEGs common to shoot and root tissue may be involved in translation (upregulated) or photosynthesis, generation of precursor metabolites and energy (downregulated) (Supplementary Fig. 1; Supplementary Data 3).

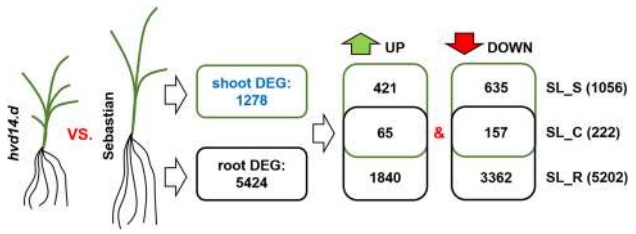


Fig. 2 Overview of differentially expressed genes (DEGs) identified in shoot and root tissues when compared SL-insensitive barley mutant *hvd14.d* and its parent variety Sebastian (wild-type). SL_C—SL-related common genes; SL_S—SL-specific shoot DEGs; SL_R—SL-specific root DEGs

Shoot-specific DEG analysis

Among the SL_S upregulated DEGs, enriched BP GO terms were related to RNA metabolism and processing, in addition to peptide biosynthesis and phosphorylation. Again, this is consistent with a ribosomal, mitochondrial and nuclear cellular localizations. Whereas downregulated SL_S DEGs were involved in cell wall organization and biosynthesis, enriched in an apoplast/cell wall and cytoskeletal cellular localization (Supplementary Data 2).

Root-specific DEG analysis

Lastly, we find that GO-enriched terms for roots were the most diverse, with upregulated SL_R DEGs enriched for peptide metabolism and response to various stimuli and stresses. Conversely, downregulated SL_R DEGs are enriched for genes involved in BP phosphorylation, cell communication, transport or response to the stimulus, while the CC-enriched terms included nuclear, plastidial or cell wall localizations (Supplementary Data 2). SL-dependent DEGs specific to only shoots or roots were more diverse regarding upper-hierarchy GO-terms. Moreover, those GO-terms do not coincide with those GO-terms described for the SL-dependent common DEG (Supplementary Fig. 1). This is another indication that the role of SLs in plant development depends on the type of tissue where they are active.

Prediction of SL-related transcription factors (TF)

Given the substantial transcriptome changes we found in our study, we queried our dataset for potential transcription factors. We find that 6% (390) of the DEGs identified were TFs (Supplementary Data 4). The highest number of TFs was found among the SL_R (root-specific) DEGs, where we found 321 TFs. This result is related to the high number of genes in this category because TF still account for 6% (321/5202), the same as in SL_C (common) (5.8%; 13/202) and SL_S (shoot-specific) (5.3%; 56/1056). By comparing Sebastian to *hvd14.d*, we can deduce which subset of these TFs may be related to SL signal transduction (TF_SL_DEG). For each 390 TF putatively involved in SL signalling, we identified the *A. thaliana* homologue (Supplementary Data 4), and within those homologues, six genes (AT1G09530, AT4G25560, AT2G02450, AT5G25190, AT3G16770, AT3G22830) were previously reported as SL-responsive (Wang et al. 2020).

Next, the promoter sequences (1500 bp) of each identified DEG were screened to find the TF binding sequences that regulate the transcription of those genes—TF_SL_PROM (Supplementary Data 5). This analysis showed that 65 identified above TF_SL_DEG recognize promoter binding sites in the DEG identified here (Supplementary Data 4). Finally,

shoot and root DEG lists were used to identify those TFs that are proposed to bind to the most represented promoter binding elements and therefore may regulate DEG expression. Those analyses allowed to type the 87 TF with significantly over-represented targets in DEG lists (30 – SL_C, 13 – SL_S, 44 – SL_R) (Supplementary Data 6).

Combining all previously described analyses, we were able to select 28 genes, which were (1) differentially expressed in *hvd14.d* vs Sebastian comparison (SL_DEG), (2) identified as TFs (TF_SL_DEG), (3) suggested to recognize TF motifs in the promoter region of other DEGs, and (4) motifs recognized by those TFs that are the most abundant among the DEG promoters (Table 1, Supplementary Data 7). No DEG from the SL_C category (differentially expressed in both shoot and root) was found among them. Whereas six and 22 TFs were found among shoot- and root-specific DEG populations, respectively. It has to be highlighted that all 28 TFs may recognize the targets that belong to each of the identified DEG categories: SL_C, SL_S and SL_R (Supplementary Data 7).

Relational analysis of identified TFs using association networks

Next, to better contextualize our identified TFs, we used STRING-DB (Szklarczyk et al. 2023) to perform an association network analysis of the shoot and root TFs. We were able to link three groups of SL-dependent TFs, which interact with each other (Fig. 3, Supplementary Data 8). The largest network identified comprises 12 proteins (42% of all identified TFs), including seven TFs belonging to the WRKY family. GO analyses revealed that identified TFs are involved, i.e. in the regulation of cutin biosynthetic, camalexin biosynthesis, response to ethylene and salicylic acid, regulation of leaf senescence or lateral root development (Fig. 3, Supplementary Data 8).

SLs induce expression of HORVU.MOREX.r2.1HG0041130.1 in HvD14-dependent manner

Among potential SL-dependent TFs which are active in barley shoots, the biggest difference in expression between *hvd14.d* and Sebastian (2.17 $\log_2$FC) was observed for HORVU.MOREX.r2.1HG0041130 (*A. thaliana* homologue: AT4G17980.1) (Table 1). In previous studies, the increased expression of this gene was observed in Sebastian (4.17 $\log_2$FC), but not in *d14*, during a response to drought (Daszkowska-Golec et al. 2023). To test the role of SLs in the control of HORVU.MOREX.r2.1HG0041130 expression, the SL spraying experiments on 2-week-old Sebastian and *hvd14.d* seedlings were performed. Two concentrations (1 and 10 µM) of synthetic SL analogue GR24 and a mock solution (0.01% acetone) were used. Before treatment, there were no differences in the expression of HORVU.MOREX.r2.1HG0041130 in the shoot of Sebastian and *hvd14.d* 2-week-old seedlings (Fig. 4). Thirty minutes after treatment, both SL concentrations do not alert the expression of HORVU.MOREX.r2.1HG0041130 in analyzed genotypes compared to the control plants sprayed with the mock solution. However, 1 h after treatment, expression of the investigated gene was induced by 1 and 10 µM of GR24 only in Sebastian. Finally, 3 h after treatment, induced expression was noted only in Sebastian seedlings sprayed with lower GR24 concentration (Fig. 4). The obtained data indicate that expression of HORVU.MOREX.r2.1HG0041130 is regulated in an HvD14-dependent manner because no effect of GR24 treatment was observed in *hvd14.d* plants. On the other hand, differences in HORVU.MOREX.r2.1HG0041130 expression observed for Sebastian seedlings at different times after treatment points out the temporal control of SLs on the expression of HORVU.MOREX.r2.1HG0041130.

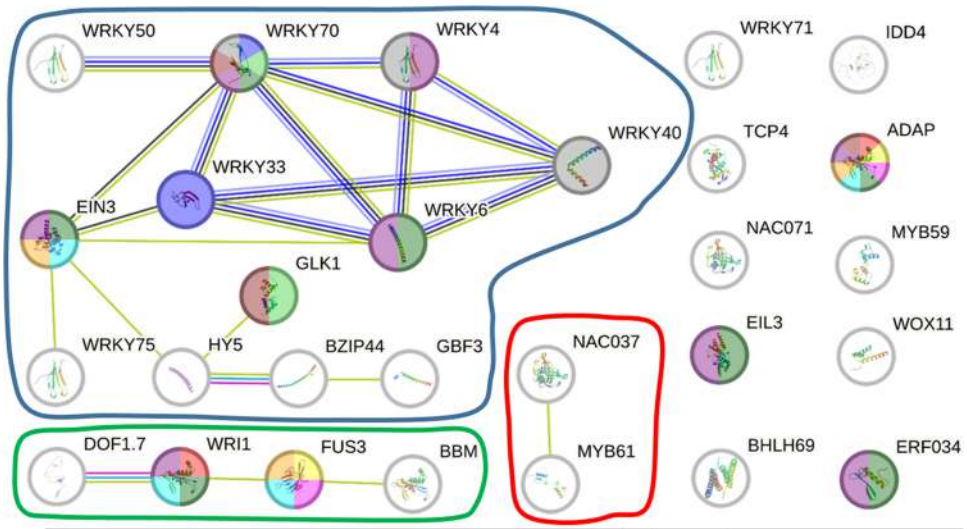
Discussion

SL insensitivity impacts the development of barley shoot and root under hydroponic conditions

SLs are known mediators of shoot and root development, crucial in plant adaptation to environmental conditions. Photoperiod and temperature (Djennane et al. 2014), light intensity and length (Jia et al. 2014), and above all, nutrient availability (Yoneyama et al. 2013) affect SL biosynthesis/signalling, which influences plant development. Thus, the phenotype of SL mutants may vary depending on growing conditions. Here, we grew barley SL-insensitive mutant *hvd14.d* for the first time under hydroponic conditions. Previous analyses carried out on plants anchored in the soil or vermiculite revealed that *hvd14.d* produces twice as many tillers as WT. However, those differences were less pronounced in the first stages of plant development, i.e. 3-week-old *hvd14.d* plants grown in the soil produce 50% more tillers than Sebastian (3 ± 0.39 and 2 ± 0.21, respectively) (Marzec et al. 2016). Hydroponic culture in ½ Hoagland solution (Hothem et al. 2003) increased the number of tillers in both genotypes at the same level. Still, the differences between genotypes are similar, and a 60% higher number of tillers was observed in *hvd14.d* (Fig. 1). Plant height was the second feature differentiating both genotypes. The height of mutant *hvd14.d* grown in soil and hydroponics was reduced by about 20%. However, both genotypes were higher when grown in hydroponics (Sebastian: 34.5 ± 1.25 vs 29.1 ± 3.7 cm; *hvd14.d*: 27.5 ± 2.32 vs 22.4 ± 4.1 cm) (Marzec et al. 2016) (Fig. 1). It was previously reported that hydroponic conditions promote shoot development when compared to

Table 1 Barley SL-dependent TFs which were identified in the presented study

	#	HORVU ID	MLOC ID	Best hit in A. thaliana	Description for the best hit	*TF_SL_DEG*	
						hvd14.d vs WT	
						log_2FC	adj.pval
SL_S	**1**	**HORVU.MOREX.r2.1HG0041130.1**	MLOC_58950	AT4G17980.1	NAC domain-containing protein 71	2,17	1E−10
		TF involved in tissue reunion of wounded inflorescence stems; involved in the cellular response to auxin stimulus					
	2	**HORVU.MOREX.r2.7HG0557800.1**	MLOC_64612	AT1G51700.1	DOF zinc finger protein 1	−1,12	2E−03
		TF that binds specifically to a 5'-AA[AG] G-3' consensus core sequence; involved in metal ion binding and response to chitin					
	3	**HORVU.MOREX.r2.6HG0479210.1**	MLOC_52112	AT5G11260.1	bZIP family protein	1,13	8E−13
		TF that promotes photomorphogenesis in light; involved in response to abscisic acid and response to karrikin					
	4	**HORVU.MOREX.r2.1HG0014470.1**	MLOC_59663	AT1G09540.1	myb domain protein 61	−3,33	4E−03
		Functions as a transcriptional regulator of stomatal closure; involved in vasculature development					
	5	**HORVU.MOREX.r2.1HG0046030.1**	MLOC_3095	AT2G44940.1	ERF family protein	−2,64	4E−07
		Involved in ethylene-activated signalling pathway					
	6	**HORVU.MOREX.r2.5HG0399940.1**	MLOC_70754	AT1G16060.1	ARIA-interacting double AP2 domain protein	−1,52	2E−03
		Involved in response to water deprivation and response to abscisic acid; positive regulation of the fatty acid biosynthetic process					
SL_R	**7**	**HORVU.MOREX.r2.2HG0148630.1**	MLOC_70754	AT5G17430.1	AP2 family protein	1,03	3E−06
		Regulation of transcription, DNA-templated					
	8	**HORVU.MOREX.r2.7HG0539480.1**	MLOC_70754	AT3G54320.1	AP2 family protein	−5,86	3E−04
		TF involved in response to sucrose; ethylene-activated signalling pathway; positive regulation of cutin biosynthetic process					
	9	**HORVU.MOREX.r2.6HG0499980.1**	MLOC_60958	AT2G02080.1	indeterminate(ID)-domain 4	−1,01	3E−13
		TF that may act as a transcriptional activator of nuclear-encoded photosynthetic gene expression					
	10	**HORVU.MOREX.r2.2HG0166600.1**	MLOC_66134	AT3G56400.1	WRKY DNA-binding protein 70	−1,32	2E−19
		TF involved in senescence, biotic and abiotic stress responses by modulating various phytohormones signalling pathways					
	11	**HORVU.MOREX.r2.3HG0230420.1**	MLOC_54606	AT1G29860.1	WRKY DNA-binding protein 71	−1,05	3E−10
		Regulation of transcription, DNA-templated					
	12	**HORVU.MOREX.r2.5HG0406860.1**	MLOC_10823	AT1G73730.1	ETHYLENE-INSENSITIVE3-like 3	−1,06	4E−10
		Encodes a putative TF involved in ethylene signalling					
	13	**HORVU.MOREX.r2.3HG0203860.1**	MLOC_68285	AT3G15030.2	TCP family protein	1,04	6E−03
		TF playing a pivotal role in the control of morphogenesis of shoot organs by negatively regulating the expression of boundary-specific genes					
	14	**HORVU.MOREX.r2.3HG0209060.1**	MLOC_68299	AT1G62300.1	WRKY family protein	−1,20	4E−23
		TF involved in response to low phosphate stress					
	15	**HORVU.MOREX.r2.4HG0342310.1**	MLOC_56769	AT2G18060.1	vascular-related NAC-domain protein 1	1,09	3E−03
		Expressed in root metaxylem pole and in shoot pre-procambium and procambium					
	16	**HORVU.MOREX.r2.3HG0236170.1**	MLOC_15725	AT3G26790.1	B3 family protein	2,78	1E−04
		Positive regulation of abscisic acid biosynthetic process					
	17	**HORVU.MOREX.r2.5HG0400770.1**	MLOC_13438	AT4G30980.1	LJRHL1-like 2	1,15	5E−15
		Involved in root hair elongation					
	18	**HORVU.MOREX.r2.6HG0471210.1**	MLOC_60890	AT1G80840.1	WRKY DNA-binding protein 40	−1,48	3E−27
		Involved in response to various phytohormones					
	19	**HORVU.MOREX.r2.5HG0371550.1**	MLOC_69575	AT1G13960.1	WRKY DNA-binding protein 4	−1,61	1E−26
		Involved in response to various phytohormones					
	20	**HORVU.MOREX.r2.4HG0315980.1**	MLOC_65745	AT3G03660.1	WUSCHEL-related homeobox 11	−3,49	3E−07
		TF involved adventitious root development					
	21	**HORVU.MOREX.r2.4HG0316110.1**	MLOC_14619	AT3G20770.1	EIL family protein	−1,34	8E−71
		TF involved in response to hypoxia					
	22	**HORVU.MOREX.r2.1HG0074290.1**	MLOC_6711	AT2G46270.1	G-box-binding factor 3	−1,16	3E−11
		Response to abscisic acid					
	23	**HORVU.MOREX.r2.3HG0207800.1**	MLOC_65876	AT2G20570.1	GBF's pro-rich region-interacting factor 1	−1,96	3E−06
		Positive regulation of organelle organization					
	24	**HORVU.MOREX.r2.6HG0460660.1**	MLOC_63436	AT1G75390.1	basic leucine-zipper 44	1,06	3E−07
		Regulation of transcription, DNA-templated					
	25	**HORVU.MOREX.r2.1HG0023190.1**	MLOC_12079	AT5G26170.1	WRKY DNA-binding protein 50	−3,30	6E−21
		TF involved in jasmonic acid-mediated signalling pathway					
	26	**HORVU.MOREX.r2.5HG0366230.1**	MLOC_52439	AT5G59780.3	myb domain protein 59	1,29	4E−19
		Involved in response to various phytohormones					
	27	**HORVU.MOREX.r2.3HG0254080.1**	MLOC_67851	AT2G38470.1	WRKY DNA-binding protein 33	−2,22	1E−66
		TF involved in defense responses					
	28	**HORVU.MOREX.r2.2HG0079820.1**	MLOC_36657	AT5G13080.1	WRKY DNA-binding protein 75	−2,21	3E−18
		TF involved in lateral root development					



term description	observed gene	background gene	strength	fdr
Positive regulation of cutin biosynthetic proc.	2	3	2,82	0.0013
Camalexin biosynthetic process	2	11	2,25	0.0069
Negative regulation of leaf senescence	2	16	2,09	0.0110
Positive regulation of lipid biosynthetic proc.	2	23	1,93	0.0203
Positive reg. of small molecule metabolic	2	25	1,89	0.0234
Ethylene-activated signaling pathway	7	194	1,55	3.01e-07
Regulation of small molecule metabolic proc.	4	121	1,51	0.0010
Regulation of cellular ketone metabolic proc.	3	90	1,51	0.0089
Response to ethylene	8	273	1,46	8.99e-08
Negative regulation of developmental proc.	3	151	1,29	0.0326
Response to salicylic acid	3	163	1,26	0.0397

Fig. 3 Protein-network analysis of SL-dependent TFs, performed using STRING Database. **A** Three networks of SL-dependent TFs of known or predicted interactions were identified. **B** Gene ontology enrichment analyses revealed the biological processes in which identified SL-dependent TFs might be involved; fdr, false discovery rate. Protein-protein interactions are presented as known interactions (experimentally determined: pink lines; from curated databases: light-blue line); predicted interactions (based on: gene co-occurrence: dark-blue; gene neighbourhood: dark-green), based on the co-expression (black) or text mining (light-green)

plants sown in soil (Dutta et al. 2023) that may be caused by easy access to water and nutrients in a hydroponic medium throughout development.

Under control conditions, SLs shape root architecture by promoting root elongation and inhibiting lateral root development (Kapulnik et al. 2011; Ruyter-Spira et al. 2011). Similar results were obtained when *hvd14.d* and Sebastian were grown in a solid medium (vermiculite) and watered with ½ MS medium. The mutant exhibited shorter seminal roots in a solid medium that produced more lateral branches than Sebastian (Marzec et al. 2016). Under hydroponic conditions, the same differences were observed (Fig. 1). In contrast, both genotypes in soil and hydroponic developed the same number of seminal roots, but their length was reduced, which is a well-known effect of hydroponics (Mian et al. 1993). Root elongation in the mutant was reduced by 35% under both conditions, hydroponic: 31.2 ± 1.79 vs 20.5 ± 1.74 cm and soil 47.1 ± 2.51 vs 34.8 ± 1.94 cm, respectively for Sebastian and *hvd14.d* (Marzec et al. 2016) (Fig. 1). Differences in lateral root density between genotypes were independent of growing conditions and *hvd14.d* developed a higher number of lateral roots per 1 cm of seminal root (Marzec et al. 2016) (Fig. 1). Obtained results indicate that growing conditions do not affect shoot and root differences between *hvd14.d* and its WT, as far as plants were supplemented with an optimal nutrient concentration.

Tissue-specific transcriptome alterations caused by SL insensitivity

Because mutation in barley SL receptor HvD14 affects shoot and root development, those differences become statistically significant at the third week of plant development (Fig. 1). The transcriptome of shoot and root was compared between *hvd14.d* and Sebastian, revealing 6702 DEGs. Up to 80% of all identified DEGs were found in roots (5414 among 6702). In both organs, a more significant number of transcripts, around 60%, was downregulated (Fig. 2). Among all identified DEGs, only 3% (222) were found in both shoot and root comparisons. The largest category, up to 77% of all DEGs, was root-specific. These results indicate that SLs play a more pronounced role in root development relative to shoots in 3-week-old barley plants. It might also be related to the higher complexity of the root system at this stage of plant development, such as a large number of different cell types supporting vastly different transcriptional programs as is the case in *Arabidopsis* (Shahan et al. 2022). A 3-week-old barley seedling's root system comprises seven seminal and

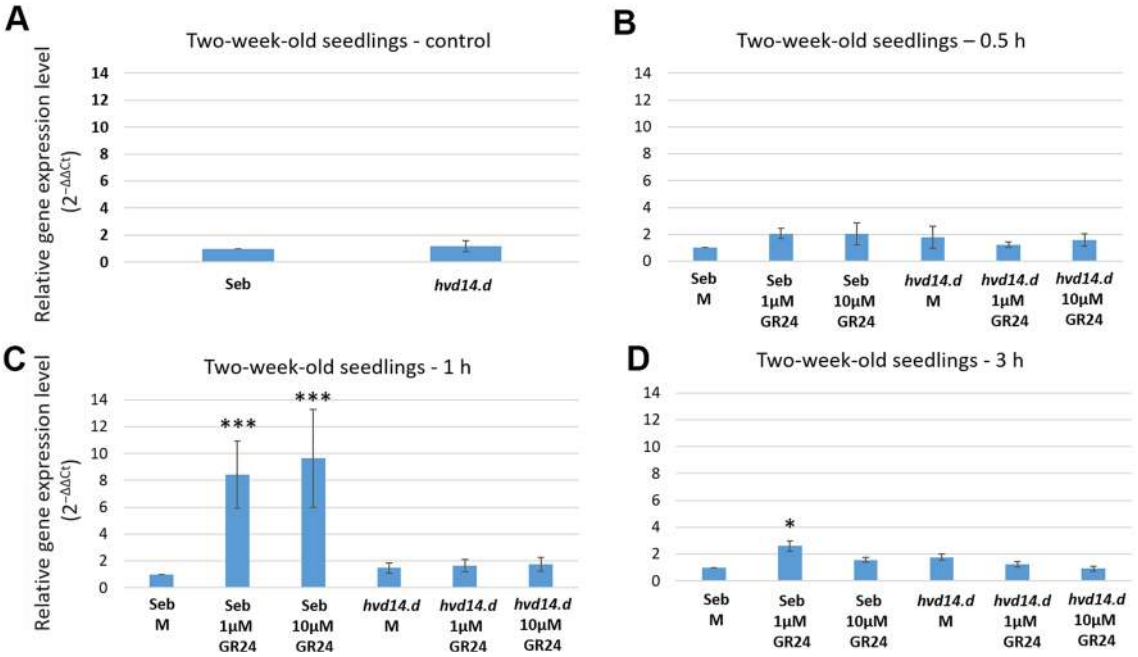


Fig. 4 Analysis of HORVU.MOREX.r2.1HG0041130 gene expression in tissues of 2-week-old seedlings of Sebastian and *hvd14.d* plants in response to GR24 treatment. **A** Relative level of expression of the HORVU.MOREX.r2.1HG0041130 gene in control (non-treated) plants **B** 30 min, **C** 1 h, and **D** 3 h after treatment with 1 plant treated with mock (0.01% acetone), 1 µM and 10 µM of $GR24^{5DS}$. Statistical analyses were performed using the *t*-test (*$p < .05$; **$p < .01$; ***$p < .001$) comparing **A** *hvd14.d* vs Sebastian or SL-treated vs mock-treated plants. Mean value with standard deviation were presented

hundreds of lateral roots at the different stages of development: initiation, elongation or branching. On the other hand, the shoot of a 3-week-old barley plant has from three to five tillers at the vegetative stage of development, which do not have developed internodes. Hence, more genes are involved in the development of the root, compared to the shoot, at this stage of plant growth. To date, there are no comparative studies about shoot and root transcriptomes for SL mutants, let alone a major crop species. Global analyses of transcriptome alterations caused by SL treatment or mutation in SL biosynthesis/signalling pathways were investigated for whole plants (Wang et al. 2020) or shoot/root separately (Zhan et al. 2018; Asghar et al. 2022; Haider et al. 2023; Li et al. 2023).

SL-responsive genes found in barley shoot and root

In total, 222 DEGs were common between the shoot and root of *hvd14.d* compared to Sebastian. Among the 65 upregulated DEGs, 25% (16) were involved in translation along with others. Moreover, DEGs involved in ribosome biogenesis (4) or RNA processing (4) were found to be upregulated in *hvd14.d* (Supplementary Data 2). It was shown that SLs increase the cold tolerance of *Brassica rapa* L. seedlings because GR24 (a synthetic analogue of SL) treatment alleviates the damage of low-temperature stress (Zhang et al. 2020a, b). Within DEG between plants pretreated with spraying 0.1 µmol·L^{-1} GR24 and non-pretreated, exposed to low temperature (4 °C), the genes encoding translation initiation factors were downregulated. On the other hand, in pea, removing apical meristem promotes the outgrowth of axillary buds, which was linked with increased expression (up to 35-fold) of gene encoding ribosomal protein (Stafstrom and Sussex 1992). The conclusion that SLs affect the translation processes via control of ribosome complex activity can be postulated. However, it cannot be excluded that stronger activity of the translational process observed in *hvd14.d* is related to the higher number of developing tillers and lateral roots, and those processes are associated with rapid protein synthesis. Hence, changes in expression of translation-related genes are not a direct result of SL activity, but the effect of SL insensitivity, resulting in the development of more meristems.

Surprisingly, both *hvd14.d* organs showed reduced gene expression related to photosynthesis and plastids (Supplementary Data 2). Changes in the expression of photosynthetic genes in non-green tissue, including roots have been widely reported for various species under different stresses, such as drought (Molina et al. 2008; Cohen et al. 2010; Ranjan and Sawant 2015; Janiak et al. 2019) or phosphate starvation (Wu et al. 2003; Li et al. 2010). It was shown that the suppression of photosynthetic genes is required for

sustained root growth of *Arabidopsis* exposed to phosphorus deficit (Kang et al. 2014). Reduction in photosynthetic genes in roots during stress responses is also linked with decreased production of reactive oxidant species (ROS) (Kang et al. 2014; Janiak et al. 2019). Our previous analyses indicated that *hvd14.d* exhibits reduced ROS scavenging under drought (Daszkowska-Golec et al. 2023). Because SL treatment seems to decrease ROS content in various species (Trasoletti et al. 2022), including barley exposed to cadmium (Qiu et al. 2021), we may speculate that SL-insensitivity of *hvd14.d* results in less efficient ROS scavenging. Thus, to reduce ROS production, the mechanisms related to photosynthesis are repressed in SL-insensitive barley mutant under control conditions. In fact, study investigating the effect of SL on photosynthesis confirm these predictions. Treatment with 1 and 5 µM of GR24 increased the net photosynthesis rate (µmol $CO_2 \cdot m^{-2} \cdot S^{-1}$) of salt stressed rice seedlings to values observed in control plants (Ling et al. 2020). Further, in cucumber (*Cucumis sativus* L.), greater photosynthetic efficiency was observed in GR24-pretreated plants than in non-GR24-pretreated plants under salt stress (Zhang et al. 2022). Under control conditions, the foliar application of GR24 on *Artemisia annua* increased various attributes related to photosynthesis (chlorophyll fluorescence, internal CO_2, and net photosynthetic rate) as well as activity of photosynthetic enzymes (carbonic anhydrase, nitrate reductase, RuBisCO) (Wani et al. 2023). The general positive role of SL on photosynthesis was well documented, so the decreased expression of photosynthesis-related genes in the shoot of SL-insensitive *hvd14.d* confirms these results. Conversely, repression of those genes in roots may be linked with reduction of processes linked to ROS production.

Shoot- and root-specific SL-responsive genes

Within upregulated DEGs described as specific for shoot tissue, the largest group among the enriched GO terms was protein phosphorylation (Supplementary Data 2). Phosphorylation is one of the main post-translational modifications that affect protein interactions and stability, hence has a significant impact on gene expression, signalling pathways and enzyme activity (Khalili et al. 2022). Chen and coworkers indicated that GR24 treatment of rice SL-biosynthesis mutant (*d10*) changed the phosphorylation status of 8 proteins at a conserved phosphorylation site (Chen et al. 2014). Upregulated DEGs in *hvd14.d* involved in phosphorylation suggest that SLs may repress phosphorylation in barley shoots. On the other hand, among downregulated shoot DEGs, the large group was annotated as related to the cell wall organization and biogenesis, cell wall polysaccharide metabolic processes or polysaccharide biosynthetic and metabolic processes (Supplementary Data 2). There is a known role for SLs in promoting secondary cell wall formation in cotton (*Gossypium hirsutum*) where exogenous GR24 increased, and the application of SL biosynthetic inhibitor (Tis108) reduced the thickness of the secondary cell wall (Wen et al. 2023). Moreover, SL biosynthesis genes (*MAX3* and *MAX4*) have been linked with xylan and cellulose deposition in *Arabidopsis* (Ramírez and Pauly 2019). Further, we previously reported the alteration of cell wall formation in *hvd14.d* in response to drought (Marzec et al. 2020). Interestingly, this is a conditional phenomenon as under control conditions; there are no differences in the cell wall thickness in the leaves of 3-week-old seedlings of *hvd14.d* and Sebastian (Marzec et al. 2020); however, there have been no investigations into the chemical composition of the cell wall to date. Thus, the differences in the polymer content between *hvd14.d* and Sebastian cannot be excluded. Secondary cell walls contain mainly cellulose, xylans and lignin, but their proportions and modifications depend on the functional needs of cell/tissue and, thus may vary between leaves and roots (Kumar et al. 2016). The data obtained, where decreased expression of genes related to cell wall biosynthesis was found specifically in barley shoot (Supplementary Data 2), narrowed down the possible role of SLs in the biosynthesis of cell wall components to that characteristic for shoot.

Within SL-related upregulated DEG found in roots, a significant number was annotated as cell cycle or cell cycle processes. It could be explained by the larger number of developing roots, thus the higher number of fast-dividing meristems in *hvd14.d* compared to the Sebastian (Fig. 1). On the other hand, both up and downregulated DEGs were annotated as involved in responses to abiotic stresses, stimuli and chemical or oxygen-containing compounds. Because SLs play an important role in plant adaptation to stresses, the insensitivity to SLs may disturb the multiple pathways related to the plant's stress responses.

SL-dependent TFs

General knowledge of the SL signalling pathway and the individual proteins involved in signal transduction is well established in model species such as *Arabidopsis* or rice, from the SL signal perception to the degradation of the SL repressor (Marzec and Brewer 2019). However, we still have rudimentary information about the transcriptional responses in crops and non-model plants. Particularly the TFs that regulate the plant's response to SLs. Here, by simultaneously comparing changes in the shoot and root transcriptome of *hvd14.d* and Sebastian, we proposed a set of TFs that may play a role in SL signal transduction in barley and which are involved in phenotypic changes observed in the shoot and root architecture of 3-week-old plants described above. In total, 28 TFs were identified as putative SL-related TFs as they (1) exhibit changed expression in *hvd14.d* versus

Sebastian, (2) they are proposed to recognize binding sites in promoters of a multitude of identified DEGs and (3) motifs recognized by those TFs are over-represented (p-value ≤ 0.05) in DEG promoters (Table 1, Supplementary Data 9).

Interestingly, no one TF was differentially expressed in shoot and root barley tissue (SL_C), indicating differences exist in SL signal transduction between these two organs. Four TFs were previously identified as putatively involved in mediated SL-dependent barley response to drought (Daszkowska, 2023) (Supplementary Fig. 2). Two of these HORVU.MOREX.r2.6HG0471210.1 (AT1G80840) and HORVU.MOREX.r2.1HG0074290.1 (AT2G46270) are involved in plant response to abscisic acid (ABA) and were found to be upregulated by drought only in the Sebastian shoot. At the same time, under control conditions, their expression was downregulated in *hvd14.d* root relative to Sebastian (Daszkowska-Golec et al. 2023). It was previously shown that ABA may regulate lateral root formation (De Smet et al. 2003; Orman-Ligeza et al. 2018). However, the interactions between SLs and ABA have been described in various aspects of plant development under both control and stress conditions (Korek and Marzec 2023). Thus, we may conclude that the higher number of lateral roots observed in *hvd14.d* is related to the disorder in ABA signalling caused by the SL-insensitivity, similar to a weaker response of *hvd14.d* to drought stress (Daszkowska-Golec et al. 2023).

Another TF, HORVU.MOREX.r2.1HG0041130.1 (AT4G17980) mediates the auxin response and was upregulated in *hvd14.d* shoots. Auxin export, which is necessary for the outgrowth of axillary buds, is blocked by SLs to suppress shoot branch development (Shinohara et al. 2013). In *hvd14.d*, which develops a higher number of tillers, the increased auxin export induces auxin signalling, i.e. via expression of HORVU.MOREX.r2.1HG0041130.1. The last TF identified as SL-dependent under control and drought conditions was HORVU.MOREX.r2.3HG0209060.1 is an ortholog of WRKY6 in *Arabidopsis* (AT1G62300) and is described as being involved in response to low phosphate (Chen et al. 2009). Under phosphorus deficiency, WRKY6 binds the promoter of PHOSPHATE1 (PHO1) (Chen et al. 2009), increasing the production of lateral roots (Williamson et al. 2001). Given the observed root phenotype observed here, HORVU.MOREX.r2.3HG0209060.1 could play a broader role in SL-dependent repression of lateral root development in barley. Because SL treatment induced HORVU.MOREX.r2.1HG0041130 expression in WT plant, but not in the SL-insensitive mutant *d14* (Fig. 4), we may assume that SLs control HORVU.MOREX.r2.1HG0041130 expression in a D14-dependent manner. Moreover, it was also previously shown that in response to drought, the expression of HORVU.MOREX.r2.1HG0041130 increases in Sebastian plants, but not in the *d14* mutant (Daszkowska-Golec et al. 2023). On the other hand, exogenous GR24 induced the HORVU.MOREX.r2.1HG0041130 expression 1 (1 and 10 µM) or 3 h (1 µM) after treatment, but not after 30 min (Fig. 4). Thus, the temporal control of SLs on HORVU.MOREX.r2.1HG004113 can be postulated, which also depends on the SL concentration. Because, in older plants (3-week-old plants) grown in hydroponics, the increased expression of HORVU.MOREX.r2.1HG0041130 was observed in *d14*; the open question remains how plant developmental stage and growing conditions affect the expression of HORVU.MOREX.r2.1HG0041130

Assessment of the association between identified SL-dependent TFs revealed significantly more interactions than expected (PPI enrichment p-value:< 1.0e−16), indicating that the proteins are at least partially biologically connected. Moreover, 42% of all identified SL-dependent TFs were grouped in the single network of known and predicted interactions (Fig. 3). As expected, among all TFs, the proteins annotated as hormonal responsive were overrepresented. However, proteins involved in response to ethylene and salicylic acid were also identified, pointing out the interactions between SLs and those two phytohormones in shaping shoot and root architecture in barley. Finally, two out of three genes related to positive regulation of cutin biosynthesis were identified as SL-dependent TFs (Fig. 3). Cutin is a main component of the cuticle (Fich et al. 2016), with the biosynthesis pathway similar to other plant hydrophobic polymer suberin (Pollard et al. 2008) that accumulates in the apoplastic regions of non-cutinized boundary cell layers, such as root exodermis (Vishwanath et al. 2015). Previously, it was postulated that SLs modulate wax biosynthesis and deposition in plants (Li et al. 2020b; Marzec et al. 2020; Li et al. 2019).

Interestingly, genes controlling camalexin biosynthesis were found among SL-dependent TFs. Camalexin is one of the phytoalexins, which are the antimicrobial compounds produced by plants (Hammerschmidt 1999). SLs may play a dual role in interactions with bacteria and fungi to (1) promote the symbiosis with arbuscular mycorrhizal (AM) fungi (Kodama et al. 2022) or (2) increase the resistance against pathogen bacteria and fungi (Marzec 2016). Thus, it may be postulated that SLs control microbial interactions via camalexin synthesis. However, a new role of camalexin in controlling lateral root formation in *Arabidopsis* was recently described (Serrano-Ron et al. 2021). Up to now, a similar function of camalexin in monocots has not been reported. Still, it cannot be excluded that SL-insensitivity in barley disturbs camalexin biosynthesis, which affects lateral root development.

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Declarations

Conflict of interest The authors declare no competing interests.

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Strigolactone insensitivity affects the hormonal homeostasis in barley

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Strigolactone insensitivity affects the hormonal homeostasis in barley

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In response to environmental changes, plants continuously make architectural changes in order to optimize their growth and development. The regulation of plant branching, influenced by environmental conditions and affecting hormone balance and gene expression, is crucial for agronomic purposes due to its direct correlation with yield. Strigolactones (SL), the youngest class of phytohormones, function to shape the architecture of plants by inhibiting axillary outgrowth. Barley plants harboring the mutation in the *HvDWARF14* (*HvD14*) gene, which encodes the SL-specific receptor, produce almost twice as many tillers as wild-type (WT) Sebastian plants. Here, through hormone profiling and comparison of transcriptomic and proteomic changes between 2- and 4-week-old plants of WT and *hvd14* genotypes, we elucidate a regulatory mechanism that might affect the tillering of SL-insensitive plants. The analysis showed statistically significant increased cytokinin content and decreased auxin and abscisic acid content in 'bushy' *hvd14* compared to WT, which aligns with the commonly known actions of these hormones regarding branching regulation. Further, transcriptomic and proteomic analysis revealed a set of differentially expressed genes (DEG) and abundant proteins (DAP), among which 11.6% and 14.6% were associated with phytohormone-related processes, respectively. Bioinformatics analyses then identified a series of potential SL-dependent transcription factors (TF), which may control the differences observed in the *hvd14* transcriptome and proteome. Comparison to available *Arabidopsis thaliana* data implicates a sub-selection of these TF as being involved in the transduction of SL signal in both monocotyledonous and dicotyledonous plants.

Keywords *Hordeum vulgare*, Branching, Strigolactones, Phytohormone cross-talk

Strigolactones (SL) represent a class of plant hormones regulating various aspects of plant growth and development, including inhibiting shoot branching through intricate interactions with other hormonal pathways[1]. However, the detailed SL-related mechanism that shapes the plants' architecture, a crucial agronomic trait directly affecting the plants' yield, is still unravelled. The initial identified downstream genes, whose expression is SL-dependent, encode proteins belonging to the TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1 (TPC) family[2]. The most extensively documented member of this family in the literature is the BRANCHED1 (BRC1), which acts as a transcription factor (TF) locally in buds and regulates the shoot branching by inhibiting the axillary bud outgrowth. The Arabidopsis (*Arabidopsis thaliana*) study showed that *atbrc1* mutants display a 'bushy' phenotype, which can not be rescued by SL application[3]. Moreover, SL-insensitive and SL-depleted plants exhibited a notable decrease in the accumulation of *BRC1* transcripts[3–8]. It was shown that the expression of *BRC1* is constitutively up-regulated in plants deficient in SL-repressor proteins, while its expression is downregulated in gain-of-function SL-repressor mutants[9,10]. However, no experimental data shows that *BRC1* is a direct target of SL-repressor. Contrary, both genetic and physical interactions between rice (*Oryza sativa*) SQUAMOSA PROMOTER BINDING PROTEIN-LIKE14 (OsSPL14) and TEOSINE BRANCHED1 (OsTB1), a rice BRC1 orthologue, have been described, leading to the hypothesis that *OsTB1* transcription is regulated by OsSPL14, known in literature as a negative regulator of branching[11]. Further studies confirmed the direct interaction between SL repressor and OsSPL14, recognising SL as a key phytohormone that profoundly influences shoot architecture[11]. However, this complex regulatory network governing shoot

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branching also involves dynamic interactions between SL and other plant hormones, specifically auxins (AUX) and cytokinins (CK), orchestrating a finely tuned regulatory system.

The pivotal role of AUX in regulating shoot branching was first discovered in 1930s, when experiments showed that removing the shoot apex in plants triggered the activation and growth of axillary buds[12,13]. Conversely, the treatment of decapitated stumps with AUX suppresses bud outgrowth[14]. The AUX canalisation model assumes that AUX forms narrow transport streams that connect AUX-synthesising tissues with regions where AUX regulates diverse molecular pathways[15]. Polar AUX transport is mediated by PIN-FORMED efflux carrier proteins (PINs), with PIN1 being a crucial protein involved in the transport of AUX within the stem[16]. The phenotype of SL-insensitive or SL-depleted mutants can be explained by SL influence on AUX transport via regulating the expression and polar localization of AUX transporters. Consistent with this idea, rice and Arabidopsis SL mutants have increased AUX transport and PIN1 accumulation[17,18]. At the same time, *rac*-GR24 (a synthetic analogue of SL) can rapidly induce depletion of PIN1 from the plasma membrane of stem xylem parenchyma cells[19,20]. Moreover, expression of genes *MORE AXILLARY GROWTH 3* and *4* (*MAX3* and *4*) encoding SL-biosynthesis enzymes are positively AUX-regulated[21–24]. This suggests that AUX and SL modulate each other's levels required for the coordinated control of axillary branching. Additionally, *BRC1* is quickly downregulated after decapitation[3,25], while applying AUX can promote *BRC1* expression in buds[2,3]. These observations highlight cross-talk between AUX and SL in regulating plant architecture.

While SL and AUX act to induce the *BRC1* expression in the buds, an adverse effect on the expression of *BRC1* and its homolog has been observed after CK application. The *BRC1* transcripts levels decreased in a CK dose-dependent manner in rice[26], pea (*Pisum sativum*)[5] and *Chrysanthemum*[27], thus highlighting the antagonistic action of CK versus SL and AUX in shoot branching regulation. Moreover, the Arabidopsis *altered meristem program1* (*amp1*) mutants accumulating higher CK levels showed increased bud outgrowth resulting from reduced *BRC1* expression[28]. In addition, the knock-out of SL-regulated *SPL13* resulted in a higher accumulation of CK and transcripts levels of CK synthesis gene *ISOPENTENYL TRANSFERASES 1* (*IPT1*) in the stem nodes[29]. The result suggests that SL inhibits lateral bud growth by suppressing CK biosynthesis. In parallel, AUX controls local CK biosynthesis in the nodal stem in apical dominance[30].

Here, using SL-insensitive barley (*Hordeum vulgare*) mutant *hvd14* and its parent cultivar Sebastian, we performed a phytohormone content profiling with transcriptomic and proteomic analyses to understand the role of SL in barley development. Our analyses allowed us to describe the SL interactions with other phytohormones in shaping the barley architecture and revealed a set of TF that might be involved in SL-related regulatory mechanisms. Together, these data enhanced our understanding of SL influence on phytohormone homeostasis during barley tillering.

Results

Mutation in SL receptor promotes tillering in barley

Barley mutant *hvd14*, carried the single transition (G725A) in the *HvD14* gene (GenBank: KP069479.1), which encodes the SL receptor (HvD14; GenBank: KP069479.1), was identified from a TILLING population[31]. Identified mutation affects the protein structure (G193E) and prevents the binding of hormone molecules, which results in the SL-insensitivity of *hvd14* plants[31]. SL-insensitivity of *hvd14* plants was observed when synthetic analogues of SL, such as *rac*GR24[31] or GR24^{5DS}[32], were used. Whereas both SL analogues inhibited tillering in the wild-type (WT) Sebastian cultivar, this effect was not observed for *hvd14*[31]. Also, under control conditions, without phytohormonal treatment, a higher number of tillers was produced by *hvd14* compared to WT. Mature *hvd14* plants developed almost twice as many tillers as WT (27 ± 4.9 and 14 ± 3.3, respectively). Differences in shoot architecture become visible and statistically significant in 4-week-old plants (Fig. 1C), and plants in that age were selected for further analysis. Additionally, 2-week-old seedlings of both genotypes before the outgrowth of first tillering tiller buds (Fig. 1A) were included in all experiments.

Mutation in *HvD14* gene results in altered phytohormone content

Phytohormones can cooperate, playing antagonistic or synergistic roles, to control different aspects of plant development, with a disturbance in the biosynthesis or signalling pathways of one phytohormone affecting the action of others, manifesting as changes in their content within and/or across plant tissues. Correspondingly, we assessed the phytohormone profiles of WT and *hvd14* plants using 2- and 4-old-week plants (Supplementary Data 1). The content of multiple phytohormones, such as abscisic acid (ABA), indol-3-acetic acid (IAA), brassinosteroids (BR), cytokinins (CK), gibberellins (GAs), jasmonic acid (JA) and salicylic acid (SA), and their intermediates were measured. Only two out of six GAs were detected in barley tissue, GA6 and GA8, and no differences were observed between 2-week-old WT and *hvd14* plants, similar to SA (Fig. 2D,F) (Supplementary Data 1). In the same comparison, WT plants exhibited higher content of ABA (40.32 vs. 28.84 pmol/g FW) and IAA (482.27 vs. 344.69 pmol/g FW) compared to the *hvd14* plants (Fig. 2A,B). However, the opposite results were obtained for CK (147.71 vs. 184.88 pmol/g FW) and JA (4.28 vs. 11.17 pmol/g FW), in which lower content was noticed in WT compared to *hvd14* seedlings (Fig. 2C,E) (Supplementary Data 1). Among all eight BRs, only the 24-nor brassinolide (norBL) was detected in the tissue of 2-week-old seedlings of both genotypes but not in the 4-week-old plants of WT or *hvd14* (Supplementary Data 1). When comparing 4-week-old plants, no statistically significant differences in IAA and GA8 were observed between genotypes, while a significantly higher content of ABA (282.75 vs. 48.48 pmol/g FW), JA (13.16 vs. 7.48 pmol/g FW) and SA (632.62 vs. 159.01 pmol/g FW) was detected in WT comparing to the *hvd14*. Conversely, significantly lower amounts of CK (164.92 vs. 199.32 pmol/g FW), was observed in WT (Fig. 2C) (Supplementary Data 1). The most significant differences in phytohormone content in 2-week-old *hvd14* seedlings were found for ABA (0.72 FC), IAA (0.71 FC) and JA (2.6 FC) compared to WT (Fig. 2A,B,E). Whereas in 4-week-old plants, the most pronounced differences in

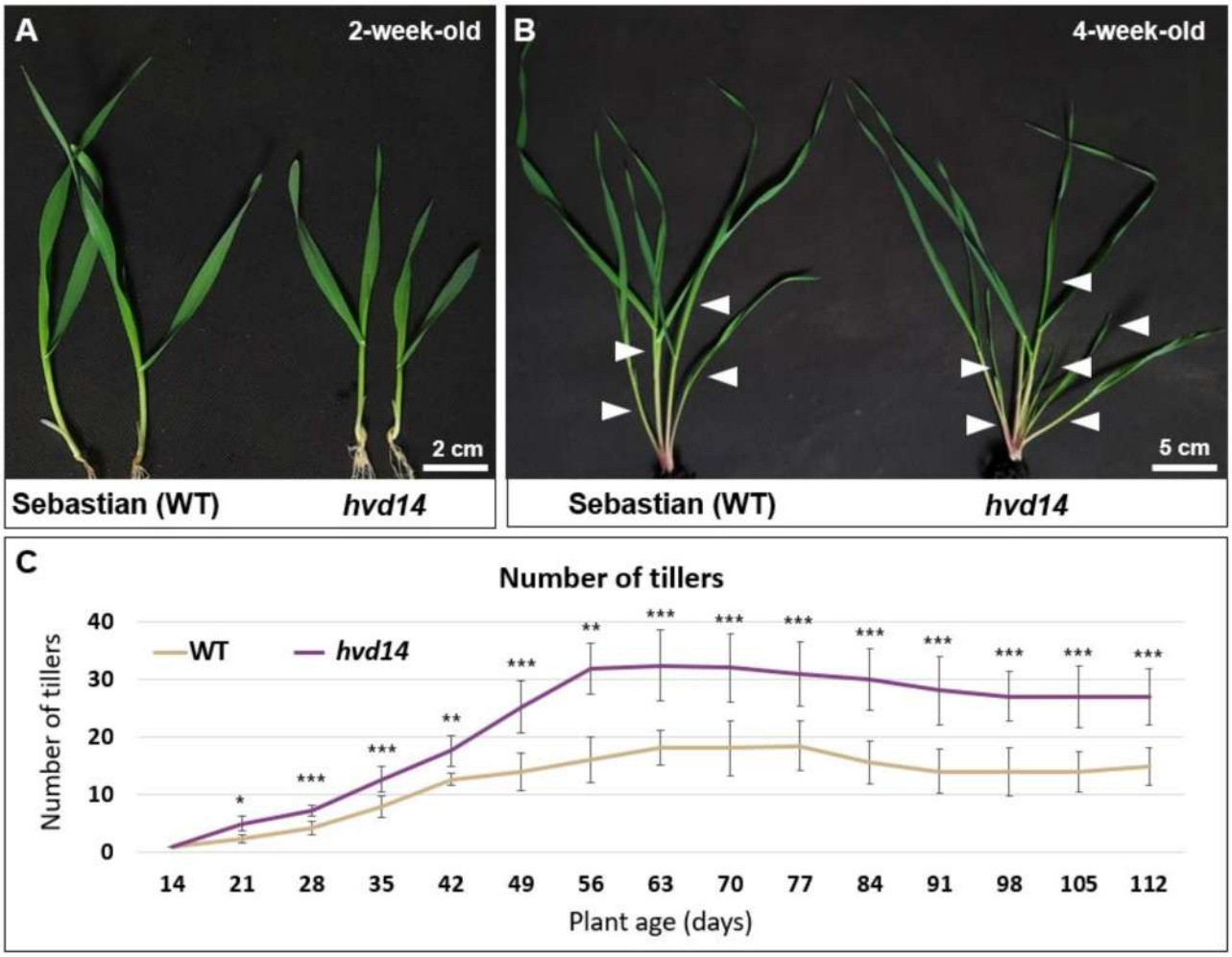


Fig. 1. The phenotype of *hvd14*. Shoot architecture of (**A**) 2- and (**B**) 4-week-old Sebastian and *hvd14*. (**C**) Differences in tillers number between WT and mutant plants across 4 months of development. The means ± SE are presented. Asterisks indicate statistically significant differences between genotypes in each time point, as determined by Student's t-test (*p*-values corresponding $*p<0.05$; $**p<0.01$; $***p<0.001$).

phytohormone composition were observed for ABA (0.17 FC), SA (0.12 FC) and JA (0.57 FC) comparing WT and *hvd14* (Fig. 2A,E,F) (Supplementary Data 1).

Because the observed differences were related to the plant age, we next assessed changes in phytohormone content related to the plant stage of development within each genotype. Here, a similar pattern of hormonal change was observed for ABA and SA, with higher accumulation found in WT vs. *hvd14*, 7.01 vs. 1.68 FC and 12.66 and 2.62 FC, respectively (Fig. 2G) (Supplementary Data 1). In contrast, IAA content decreased during development in both genotypes at a similar level (0.4 and 0.49 FC in WT and *hvd14*). Opposite trends in phytohormone content were observed for JA, which increased in WT (3.07 FC) and decreased in *hvd14* (0.67 FC), while CK and GA8 increased (1.12 FC) and decreased (0.69 FC), respectively in WT, but did not change or slighty change significantly in *hvd14* (Fig. 2G).

SL insensitivity affects transcriptome and proteome during barley development

Comparison of WT vs. *hvd14* leaf transcriptomes revealed 94 and 1120 differentially expressed genes (DEG; $\log2FC\geq1$ or $\log2FC\leq-1$, adjusted *P* value ≤0.01) for younger and older plants, respectively (Fig. 3A) (Supplementary Data 2). At both developmental timepoints, a higher number of DEG was up-regulated (54) compared to down-regulated (42) for 2-week-old plants; (620 up- and 500 down-regulated for 4-week-old plants) (Fig. 3A). Among these datasets, most of DEG were specific for either 2-week-old or 4-week-old barley plants, revealing only 30 genes in common. On the other hand, proteome analysis showed the opposite pattern, revealing more differentially abundant proteins (DAP; $\log2FC\geq0.58$ (corresponding to a 1.5-fold change) or $\log2FC\leq-0.58$, adjusted *P* value ≤0.01) for younger plants compared to older ones, which is 89 and 7, respectively (Fig. 3A), with only two DAP in common among the presented contrasting groups.

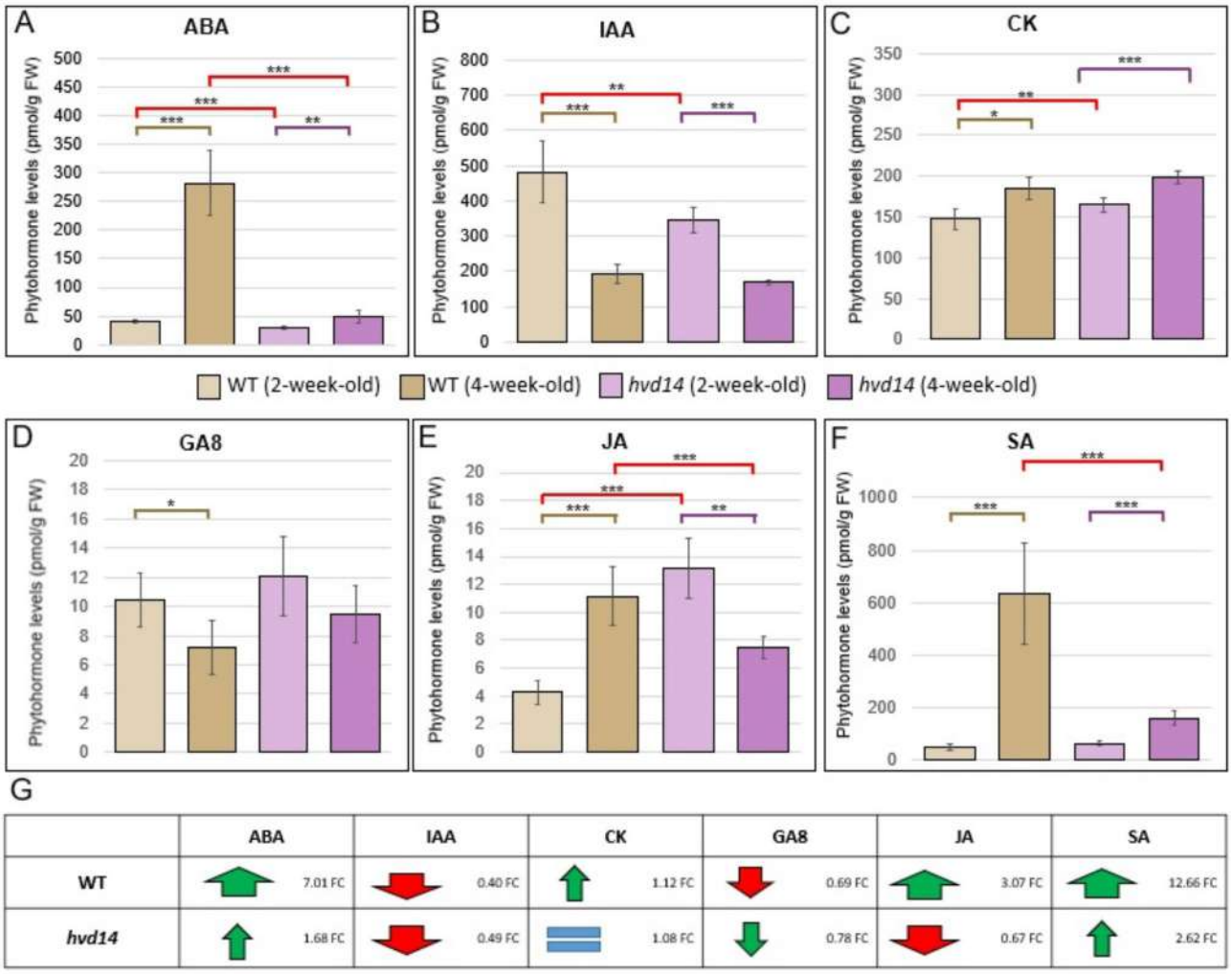
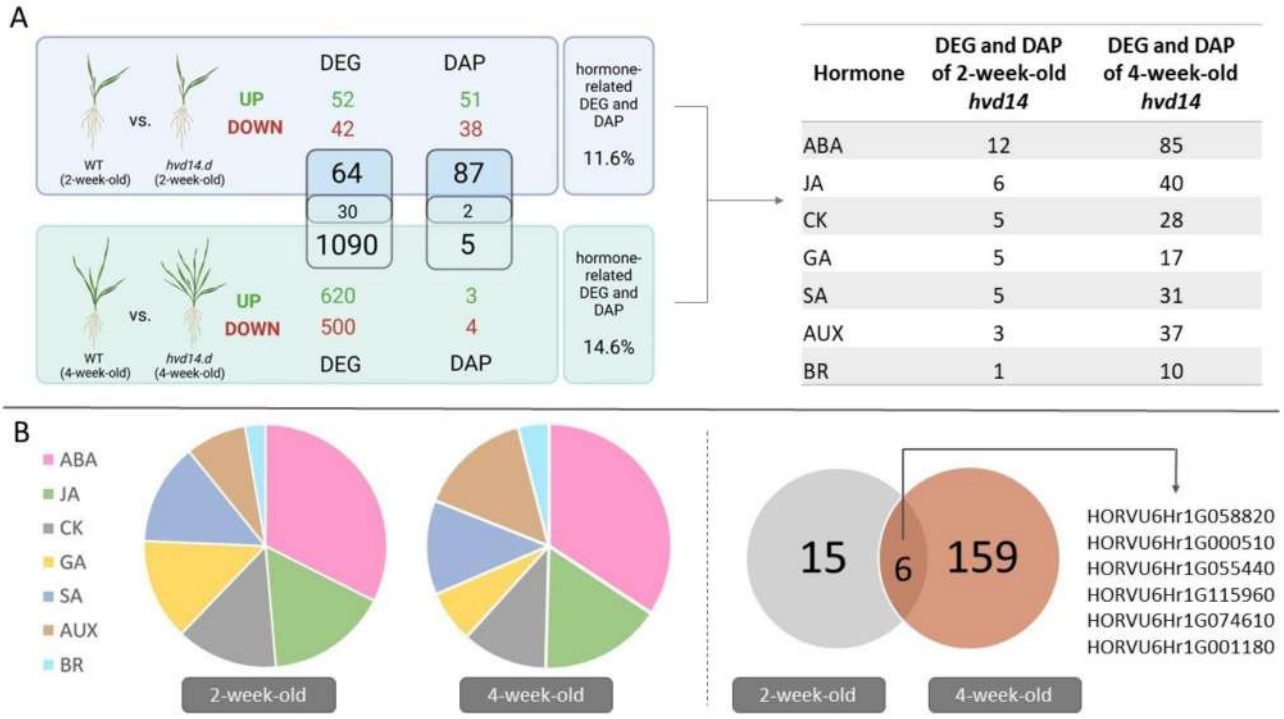


Fig. 2. Mutation in *HvD14* alters phytohormone content in barley. Measurement of (**A**) ABA, (**B**) IAA, (**C**) CK, (D) GA8, (**E**) JA, (**F**) SA content of 2- and 4-week-old Sebastian and *hvd14* plants. Asterisks indicate statistically significant differences between samples in Student's t-test (*p*-values corresponding $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). (**G**) Changes in the hormonal profiles of WT and the mutant during the early stages of plant development. The red and green arrows represent an increase or decrease in hormone content, respectively, with their size indicating the magnitude of the change. An equal sign denotes no change in the level of the analyzed hormone.

Transcriptomic and proteomic analysis reveals phytohormone-associated processes in SL-insensitive barley

The obtained DEG and DAP lists were then used to identify the transcriptome and proteome changes that may affect the mutant's hormonal balance, to uncover expression regulation mechanisms distributed in *hvd14*. Based on GO terms assigned to identified DEG and DAP, our analysis revealed that 11.6% (21/181) of transcriptomic changes and 14.6% (165/1127) of proteomic changes are associated with phytohormone-related processes for younger and older plants, respectively (Fig. 3A). However, some of the identified genes/proteins were annotated to more than one term linked to phytohormones (Supplementary Data 3). Most DEG and DAP are related to ABA and JA, which aligns with results obtained for phytohormone content measurement, where ABA and JA differences between tested genotypes were the most statistically significant. ABA and JA reflect 12/36 and 6/36 of all GO terms related to phytohormones in 2-week-old pants, as well as 85/248 and 40/248 in 4-week-old plants (Fig. 3A,B). Interestingly, the expression of six hormone-related genes was specifically regulated in the mutant at both time points tested (Fig. 3B, Table 1). Among them are three genes encoding lipoxygenases (LOX), which are associated with the production of three classes of phytohormones: ABA, JA, and SA. Specifically, our *hvd14* mutant showed a decreased content of JA, SA, and ABA, despite the increased expression of the genes encoding LOX during plants growth.

Next, we examined the promoter sequences (1500 bp) of all identified hormone-related DEG and genes encoding DAP to find TF motifs and potential over-arching regulatory TF for both 2- and 4-week-old plants (Supplementary Data 4 & 5). The prepared data allowed us to select TF that recognize binding sites in the promoter



Hormone	DEG and DAP of 2-week-old *hvd14*	DEG and DAP of 4-week-old *hvd14*
ABA	12	85
JA	6	40
CK	5	28
GA	5	17
SA	5	31
AUX	3	37
BR	1	10

Fig. 3. Transcriptome and proteome changes affected by SL-insensitivity. (**A**) The numbers of differentially expressed genes (DEG) and differentially abundant proteins (DAP) identified after the comparison analysis between 2- and 4-week-old Sebastian and *hvd14* plants. The table shows the numbers of DEG and DAP involved in phytohormone-related processes. (**B**) Pie charts showing distribution of hormone-related DEG and DAP of younger and older *hvd14*. Venn diagram shows the numbers of specific and shared hormone-related DEG and DAP (duplicates removed) for 2- and 4-week-old *hvd14* plants. The illustration was created using BioRender (www.biorender.com).

Hormone	Horvu ID	Gene description (PlantTFDB/UniProt)	2-week old		4-week old	
			log2FC	adj.pval	Log2FC	adj.pval
GA	HORVU6Hr1G058820	PHE ammonia lyase 1	− 0.63	0.0232	− 2.29	8.60E−06
ABA/JA/SA	HORVU6Hr1G000510	lipoxygenase 2	1.75	0.0499	9.04	1.37E−07
CK	HORVU6Hr1G055440	Glycine-rich RNA-binding protein 8	2.71	0.0019	4.45	0.0004
ABA/JA/SA	HORVU2Hr1G115960	PLAT/LH2 domain-containing lipoxygenase family protein	1.48	0.0063	1.65	3.82E−06
ABA/CK	HORVU2Hr1G074610	Histidine kinase 5	3.81	2.42E−06	2.94	1.61E−09
ABA/JA/SA	HORVU5Hr1G001180	lipoxygenase 2	1.14	0.0063	1.57	0.0008

Table 1. Phytohormone-related genes with altered expression in comparison of WT vs. *hvd14*, in both 2- and 4-week-old plants.

sequences of hormone-related DEG and genes encoding DAP, for both 2- and 4-week-old plants. This identified several putative TF that may regulate the expression of genes belonging to all hormone-related categories, including AUX, ABA, JA, GA, BR, CK, and SA. In total, 3 and 29 TF were selected as master regulators of phytohormonal pathways for younger and older plants, respectively (Supplementary Data 5). Interestingly, all 3 TF identified for 2-week-old plants (HORVU5Hr1G113220, HORVU2Hr1G087310, HORVU1Hr1G063610) were also identified in 4-week-old plants. Moreover, 7 TF, including HORVU5Hr1G113220 and HORVU2Hr1G087310, possess a binding site in LOX genes (Supplementary Data 5). Comparison to Arabidopsis homologues of these three genes found they encode OBF-BINDING PROTEIN 3 (OBP3), BABY BOOM (BBM) and PISTILLATA (PI) TF. OBP3 belongs to DNA BINDING WITH ONE FINGER (DOF) TF family, which is involved in a wide range of developmental processes. What is interesting is that the constitutive overexpression of many DOF TF results in plant dwarfing despite the diverse biological functions of these genes in plant growth[33,34]. Additionally, Arabidopsis transgenic lines overexpressing *OBP3* present altered root development and small, yellowish leaves[35]. Both traits are regulated by many hormone-dependent signalling pathways. However, the *OBP3* increased expression was only proved after AUX and SA treatment[35]. The second identified gene, BBM, is one of the members of the AIL/PLT (AINTEGUMENTA-LIKE/PLETHORA) family encoding TF containing an

AP2/ERF domain[36]. The presence of AIL/PLT family proteins can be observed in dividing tissues or organs, such as roots, shoots and floral meristems, where they ensure the maintenance of the meristematic state of cells[37]. Additionally, analysis of mutant collections showed that AIL/PLT proteins are dose-dependent regulators of root development. The *plt1 plt2 plt3 bbm* Arabidopsis mutant possesses completely inhibited root growth compared to WT[38]. Phenotypic data about described mutants, such as altered root system architecture—a characteristic feature of SL gene mutants—may further support their involvement in SL signaling. Additionally, it was shown that BBM transcriptionally regulates the activity of AUX-biosynthesis genes, thus promoting its accumulation in seedlings[39]. Moreover, the *BBM* overexpression in transgenic lines of Arabidopsis and *Brassica napus* results in spontaneously forming somatic embryos on seedlings without supplementation of exogenous hormones[40]. Since the embryogenic transition involves changes in hormonal homeostasis, BBM may serve as a strong candidate for an SL-related TF that influences the phytohormonal network. The last gene, PI, encodes a homeotic protein, which, together with APETALA 3 (AP3), plays a role in the formation of petals and stamens in angiosperm flowers[41]. Plants exhibiting mutation in *PI* present a male-sterile phenotype. However, the SL-depleted or SL-insensitive plants have not affected flower development, so the identification of PI as an SL-related TF cannot be excluded.

Lastly, among all detected TF that may regulate hormone-related DEG and DAP we endeavoured to sort out those TF that may bind to the most represented promoter binding elements. This allows us to predict 24 TF with over-represented targets in our dataset (Supplementary Data 6). Moreover, we compared them with 29 TF that were identified as a phytohormone master regulators, thus revealing 10 TF that may be considered as a key TF responsible for 'bushy' phenotype of *hvd14* due to phytohormones content alterations (Table 2).

Bioinformatic approach predicting SL-related TF

To better understand the molecular mechanisms underpinning the differences of WT and *hvd14* plants, we queried all of DEG and DAP data for TF. Based on amino acid sequences of all DEG and DAP, we found 8 (4.4%, 8/181) and 101 (8,9%, 101/1127) TF in younger and older plants, respectively (Supplementary Data 7). Furthermore, for each of the 109 TF, we identified an Arabidopsis ortholog, and compared obtained list with SL-responsive genes reported by Wang et al. 2020[42]. Here, the authors identified 401 potentially SL-responsive genes using ten-day-old Columbia-0 seedlings treated with 5 µM GR24^{4DO}[42]. Among them, four orthologous genes were common with our dataset (*HORVU5Hr1G000490/AT3G18550, HORVU5Hr1G068110/AT5G67060, HORVU1Hr1G090250/AT1G64380, HORVU2Hr1G028840/AT2G02820*) (Supplementary Data 7). *AT3G18550*, differentially expressed when comparing 4-week-old *hvd14* and Sebastian plants, encodes a BRC1, whose involvement in SL-related regulation of shoot branching was extensively documented, as described above. The identification of BRC1 exclusively in older and not younger plants might explain the differences in shoot phenotype, as 2-week-old barley WT and *hvd14* plants exhibited similar branching level, in contrast to 4-week-old plants. The role of the remaining three TF (*HORVU5Hr1G068110/AT5G67060, HORVU1Hr1G090250/AT1G64380, HORVU2Hr1G028840/AT2G02820*) in the signal transduction pathway has not yet been functionally tested, but the presence of motifs recognized by them in SL-dependent genes in barley and Arabidopsis indicates their significant function in this process. However, it should be emphasized that none of the 4 TF identified in

HORVU ID	Best HIT in Arabidopsis	Protein family	No. of targets in				Description (PlantTFDB/NCBI)
			2-week-old		4-week-old		
			DEG	DAP	DEG	DAP	
HORVU7Hr1G012840 (MLOC_15776)	AT5G42520	BBR-BPC	3	9	146	10	Specifically binds to GA-rich elements present in regulatory sequences of genes involved in developmental processes
HORVU6Hr1G008870 (MLOC_3855)	AT1G72050	C2H2	4	1	304	3	Required for transcription of 5 S rRNA gene
HORVU0Hr1G007050 (MLOC_24530)	AT5G44210	ERF/AP2	3	4	56	5	Protein contains one AP2 domain
HORVU2Hr1G036710 (MLOC_1876)	AT3G45260	C2H2	0	1	31	0	Functions redundantly with JACKDAW to control root development
HORVU4Hr1G070960 (MLOC_60958)	AT2G02080	C2H2	2	1	31	0	Its phosphorylation is induced under salinity stress by MPK6, regulating plant growth adaptation
HORVU5Hr1G023000 (MLOC_51930)	AT3G62420	bZIP	0	0	19	0	Forms heterodimers with group-C bZIP TF to bind to the ACTCAT cis-element of proline dehydrogenase gene
HORVU6Hr1G069190 (MLOC_73724)	AT5G62940	DOF	2	3	90	3	Induces the formation of interfascicular cambium and regulates vascular tissue development
HORVU5Hr1G018020 (MLOC_23884)	AT4G33280	AP2/B3	0	0	25	0	AP2/B3-like transcriptional factor family protein
HORVU6Hr1G017710 (MLOC_63436)	AT4G34590	bZIP	0	0	21	0	Regulates gene expression of enzyme-coding genes involved in amino acid metabolism
HORVU3Hr1G024210 (MLOC_52112)	AT5G11260	bZIP	0	0	17	0	Plays a role in anthocyanin accumulation, binds to the promoter of *ABSCISIC INSENSITIVE 5* (*ABI5*) and regulates its expression

Table 2. List of TF with over-represented targets in hormone-related DEG and DAP of 2- and 4-week-old *hvd14*.

this approach have been previously identified to control hormone-dependent differences in the transcriptome and proteome observed in *hvd14* (Supplementary Data 5 &6).

Further, we again assessed the promoter sequences in terms of identifying TF binding sites and selecting TF with over-represented targets of DEG and DAP describing differences between WT and mutant plants (Supplementary Data 8). This allowed us to predict TF with significantly over-represented targets in DEG and DAP datasets, showing that 70 and 75 TF may control the proteome and transcriptome changes in younger and older plants, respectively. Comparison of these two datasets enabled the selection of 33 common TF (Supplementary Data 9). We also identified 14 TF, which might regulate the expression of DEG and DAP, and at the same time, their abundance was altered by a mutation in the *HvD14* gene (Supplementary Data 9). These genes might be strong candidates as a master SL-responsive TF participating in SL-signal transduction. Lastly, we identified the TF with over-represented targets in promoters of SL-responsive genes reported by Wang et al. 2020[42] (Supplementary Data 10). This dataset allowed us to select 79 TF that potentially may regulate SL-responsive genes in the Arabidopsis genome.

Finally, we compare all four generated lists of TF that might be involved in SL-signalling that are: i) over-represented TF controlling expression of DEG and genes encoding DAP of 2-week-old *hvd14,* ii) over-represented TF controlling expression of DEG and genes encoding DAP of 4-week-old *hvd14,* iii) over-represented TF controlled expression of hormonal-related DEG/DAP in barley and iv) over-represented TF controlling expression of identified SL-dependent DEG in Arabidopsis (Fig. 4) (Supplementary Data 11). Ultimately, we were able to identify five TF that were common for barley and Arabidopsis in relation to SL-responses, two of which regulate expression of hormone-associated genes/proteins (Fig. 5). Those TF, HORVU7Hr1G012840/AT5G42520 and HORVU6Hr1G069190/AT5G62940, seem to be crucial in the control of SL-dependent processes that are impaired in the *hvd14*, because not only they control the expression of genes that are associated with the observed disbalance of hormonal homeostasis in the mutant, but also they control the expression of the remaining genes whose expression patterns are altered in the SL-insensitive plant. Finally, both TF may have a similar function in Arabidopsis, which means that they may be involved in SL signal transduction and SL cross-talk with other phytohormones in both mono- and dicots.

Discussion

SL-insensitivity affects barley shoot architecture

The development of branches increases the number of reproductive structures, such as flowers and fruit-bearing sites, contributing significantly to overall crop productivity[43]. Proper crop branching influences the quantity and quality of the harvest, as it ensures optimal light interception, allowing for more efficient photosynthesis or distribution of nutrients[44]. The primary phytohormone that regulates the plants' shoot architecture by inhibiting the axillary bud outgrowth is SL[45]. Thus, the SL-insensitive or SL-depleted plants possess more tillers. Our barley mutant *hvd14*, harbouring the mutation in the SL receptor, developed a higher number of tillers than WT (Fig. 1). The differences were most noticeable in 2-month-old plants, with this continuing through the remainder of the plant's development period, suggesting that this phase of plant growth is the most critical regarding tiller formation. A similar pattern was observed in our previous analysis, where 3-week-old mutant plants produced

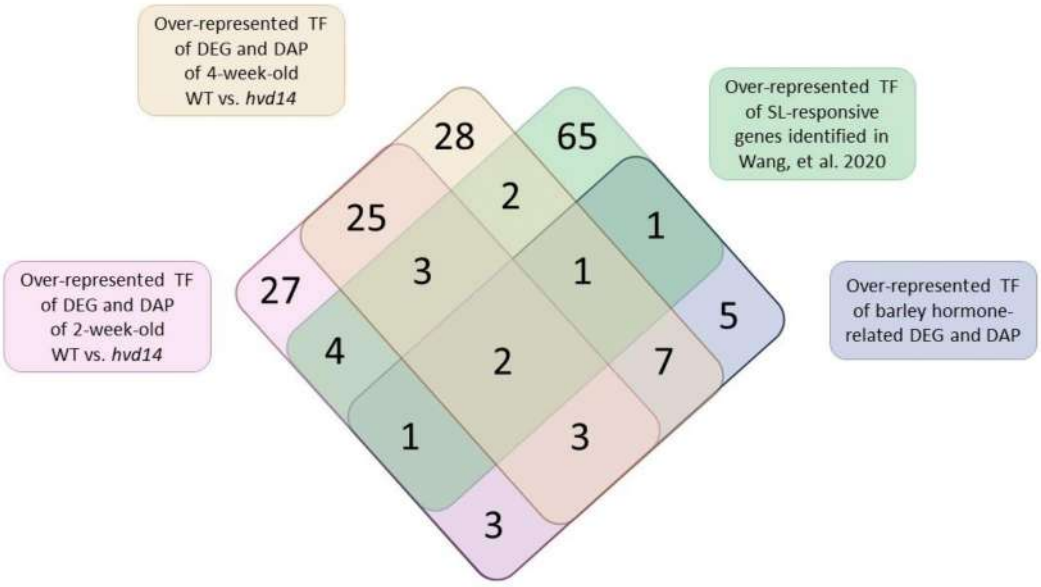


Fig. 4. TF with over-represented targets in SL-responsive genes of barley and Arabidopsis. Venn diagram showing numbers of identified SL-responsive TF specific for 2- and 4-week old WT vs. *hvd14* and Arabidopsis SL-responsive genes selected by Wang et al. 2020. The illustration was created using BioRender (www.biorender.com).

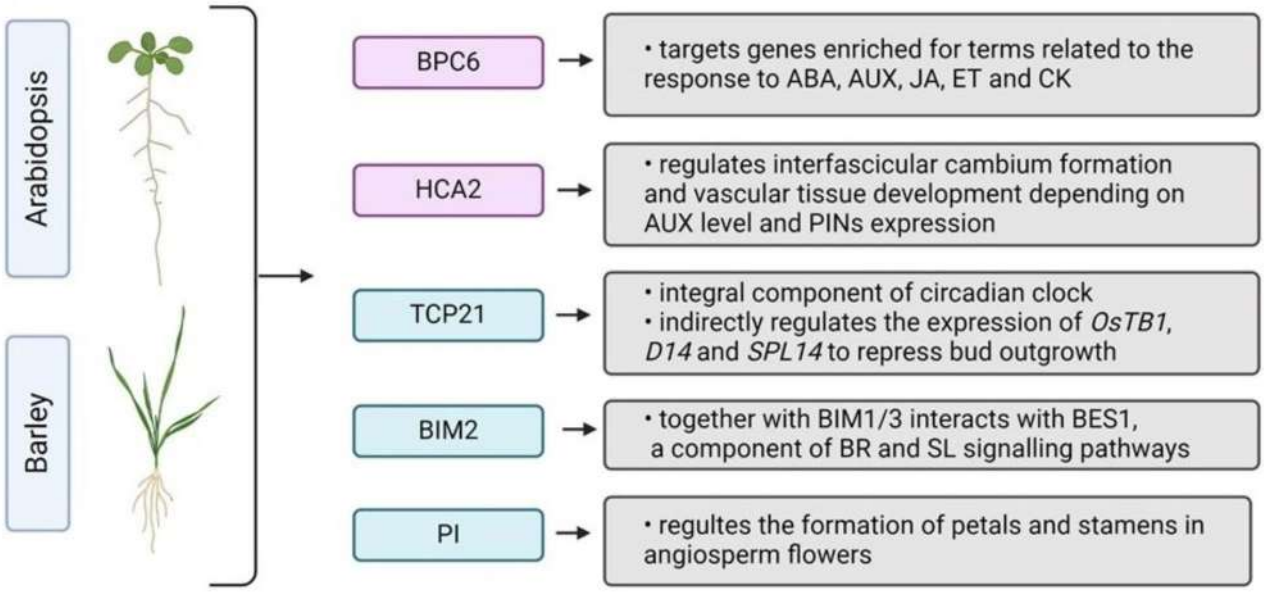


Fig. 5. TF with over-represented targets in SL-responsive genes common for barley and Arabidopsis. Pink boxes indicate TF that target genes associated with phytohormonal processes. The illustration was created using BioRender (www.biorender.com).

50% more tillers than WT, while older plants almost doubled the number of tillers[31]. In hydroponic culture conditions, this changed slightly in favor of *hvd14*, where the SL-insensitive mutant had 60% more tillers compared to WT three-week-old plants[46]. The differences in tiller formation between plants growing in soil or hydroponics conditions might be due to easier access to water and micronutrients. However, the mutation in the gene *HvD14* encoding the SL receptor which has a beneficial effect on the branching level of barley shoots.

The phenotype of the highly branched SL-insensitive mutant is the result of a fine-tuned network of hormone interactions

Extensive investigation into plant hormone levels and corresponding transcriptional changes in biosynthetic and signalling genes reveals that hormones and corresponding signalling events seem to operate as interconnected networks, with these multi-level and multi-complex hormone interactions affect the vegetative, generative, and plant defence processes in plant life cycle[47]. Thus, mutation in the *HvD14* SL receptor gene prevents SL detection within the plants disturbing the hormone homeostasis of other phytohormones in barley.

The most significant changes in phytohormone content between WT and *hvd14* plants concern ABA and JA, both in the case 2- and 4-week-old barley plants (Fig. 2A,E) (Supplementary Data 2). The lack of functionality of the HvD14 protein contributed to the reduction of ABA levels compared to WT in barley leaves, both in younger and older plants. However, these differences are much more pronounced in older plants, probably related to the different branching level in 2- and 4-week-old mutant plants. Indeed, highly-branched mutants, such as Arabidopsis *max2* and *brc1,* present decreased content of ABA in buds[48]. Partial suppression of branch elongation in these mutants by ABA treatment suggests that ABA may act downstream of SL core signalling pathway. This hypothesis is supported by the fact that *BRC1* expression was not altered after the ABA treatments. Additionally, the ABA biosynthesis mutants *9-cis-epoxycarotenoid dioxygenase 3* (*nced3*) and *aba deficient 2* (*aba2*) exhibited enhanced branching properties, suggesting a potential direct involvement of ABA in the suppression of bud outgrowth. The dissection of Arabidopsis bud into stem, young leaves, young flowers, primary shoot apex and secondary bud tissues showed that ABA accumulates mainly in meristematic tissue, highlighting its role in branching[48]. Indeed, BRC1 binds to and positively regulates the expression of *HOMEOBOX PROTEIN 21* (*HB21*), *HOMEOBOX PROTEIN 40* (*HB40*) and *HOMEOBOX PROTEIN 53* (*HB53*), which together with *BRC1* enhance *NCED3* transcription[49]. It was shown that these three homeobox proteins act in Arabidopsis axillary buds, leading to the ABA accumulation and suppression of bud development. Thus, the highly branched phenotype of our 4-week-old *hvd14* mutant may be caused by impaired cross-talk between SL and ABA pathways.

The second hormone with the most abundant differences in content between WT and *hvd14* plants is JA. The 2-week-old plants showed increased JA content in favour of mutant plants, however this relation changed with the growth and enhanced branching of the mutant (Fig. 2E) (Supplementary Data 2). Four-week-old *hv14* showed reduced JA level compared to WT. JA is known for its involvement in wound healing, plant defence responses and development of flowers. However, recent papers also point to JA involvement in shoot branching. The pear (*Pyrus communis L.)* mutant exhibiting more branched and reduced height phenotype showed significantly higher JA content than parent variety[50]. Additionally, in response to treatment with methyl-JA, the WT phenotype developed fewer branches. A similar situation was observed in identifying LITTLE NINJA (LNJ), a NINJA-related micro-protein that modulates JA signalling by attenuating the repression of JA-signalling[51].

Ectopic expression of *LNJ* in Arabidopsis resulted in dwarf height with branched shoots plants. This effect was transferable between grass species, including barley, rice, and Brachypodium, with the maintenance of high tillering of plants. However, the authors consider this JA-tillering relation to be a consequence of disturbances in the general hormone homeostasis. Hormone profiling of 'bushy' transgenic plants revealed altered JA, AUX and CK levels compared to WT. Thus, the altered JA content in our *hvd14* plants might result from cross-talk of JA with other phytohormones.

We also found that mutation in the SL receptor significantly influenced the CK and AUX content (Fig. 2B,C) (Supplementary Data 2). The CK and AUX are considered the main phytohormones that regulate shoot branching[15]. It has been proved that CK promotes bud outgrowths, while AUX acts antagonistically, inhibiting the formation of lateral branches[52]. However, with increasing reports of tillering regulation by SL, we should consider AUX-CK-SL as a critical signalling trio. The *IDEAL PLANT ARCHITECTURE 1* (*IPA1*), also known as *SPL14*, is a direct downstream component of SL-repressor protein in regulating the tiller number in rice[11]. On the other hand, recent research showed that SPL13, a downstream component of SL-signalling, controls CK biosynthesis and affects lateral bud outgrowth[29]. In tomato SL-deficient mutants the expression of *SPL13* is decreased, while the treatment with GR24 results in elevated levels of *SPL13* transcripts. Moreover, knock-out of *SPL13* by CRISPR/Cas9 technique resulted in enhanced growth of lateral buds with higher content of CK and transcripts of *ISOPENTENYL TRANSFERASES 1* (*IPT1*), a CK biosynthesis gene. Additionally, GR24 treatment suppressed CK synthesis and branching of SL-biosynthesis mutants, which was not detected in *spl13* plants. These results demonstrate that *SPL13* acts downstream in SL-signalling pathway to inhibit lateral bud outgrowth by suppression of CK synthesis[29]. It might explain why our barley SL-insensitive and 'bushy' mutant presents elevated CK content during development. This hypothesis is supported by the observation that SL and CK act antagonistically on bud outgrowth control, potentially acting on a common target. The treatment of WT pea with GR24 or BA (a synthetic analogue of CK) results in up-regulation or down-regulation of *PsBRC1* gene, respectively, while also affecting plants phenotype[5,6].

In contrast, AUX content in *hvd14* decreased at the early stages of development compared to WT (Fig. 2B) (Supplementary Data 2). On the other hand, the 4-week-old *hvd14* showed reduced AUX content compared to non-branched younger mutant plants. The SL-AUX model in the regulation of shoot branching assumes that SL regulates the expression of AUX transporters, leading to the increased content of AUX in buds, thus inhibiting its outgrowth[17]. Indeed, in our previous analysis we showed that GR24 treatment of Sebastian plants resulted in increased content of AUX in axillary buds[31]. Analogous observations were noted in the case of different mono- and dicots species[26,53,54]. Additionally, the GR24 treatment resulted in a significant elevation in the amount of AUX in rice nodes and decreased level of *PINs* genes[54]. On the other hand, the NAA treatment reduced the expression of CK biosynthesis genes and increased the expression of *OsD* genes locally in buds, highlighting that CK-AUX-SL cross-talk plays a key role in the regulation of branching. Our highly branched SL-insensitive plants showed altered content of both CK (up-regulation) and AUX (down-regulation), which coincides with the studies presented above and the generally known antagonistic relationships between CK and AUX in the regulation of bud outgrowth.

The last hormone profile affected by the mutation in the *HvD14* gene is SA. SA, similar to JA, is known for its involvement in plant defence responses against biotic and biotic stresses[55]. So far, there is very little research that points to participation of SA in branching. However, in independent research, the increased number of branching was observed in the combination of SA with ascorbic acid[56] or chelated zinc[57], in the case of Roselle (*Hibiscus sabdariffa L.)* or sweet pepper (*Capsicum annuum L.*), respectively. On the other hand, the treatment with SA alone of coriander (*Coriandrum sativum*) in field conditions does not affect the number of developing branches[58]. Therefore, due to the lack of direct reports on the involvement of SA in branching, we assume that the altered content of SA in our barley *hvd14* mutant results from disturbed homeostasis of the entire phytohormonal network.

Transcriptome and proteome changes in *hvd14* correspond with altered hormone homeostasis

Our study, combining transcriptomic and proteomic analyses, revealed various differences contributing to the distinct phenotype between WT and *hvd14* plants. The number of identified DEG was higher in 4-week-old plants, potentially due to more advanced developmental state in addition to the observed phenotype differences in branching. Moreover, only 30 DEG were common to both 2- and 4-week-old plants, indicating that the biological processes occurring in barley plant development are dynamic and development specific (Fig. 3A).

Despite measuring substantial DEG changes that increased with development, we found very comparatively fewer DAPs in either both 2- and 4-week-old plants. This suggests a number of interesting possibilities, including protein turnover, which is not specifically captured by our quantitative proteomic analysis approach, but has been suggested to be a contributing factor to the regularly observed disconnect between a significantly changing transcriptome and an unchanging proteome[59]. Our data highlights this possibility through our measurement of multiple LOXs at both the transcriptomic and proteomic level (Table 1) (Supplementary Data 2 & 3), which in Arabidopsis rosettes been shown to undergo rapid protein turnover[59]. Literature data also indicate the involvement of LOX-like enzymes in the biosynthesis of ABA[60], being more highly expressed under stressful conditions, so elevated concentrations of plant defence hormones can trigger signal transduction, including SA, JA and ABA, leading to the plant's response to adverse environmental conditions[36–40]. Here, our 2-week-old plants demonstrated a concurrent and significant transcripts and protein-level changes in two LOX2-like proteins (HORVU6Hr1G000510 & HORVU5Hr1G001180) in WT vs. *hvd14* plants, along with significant protein-level change in LOX1-like protein (HORVU4Hr1G005920). However, by 4-week-old plants, we only still see an up-regulation of transcripts, with no measurable change in LOX2 protein abundance. How these sorts of transcript-protein relationships through events such as protein turnover specifically relates to the developmental

differences between WT and *hvd14* plants represents an interesting possibility but is ultimately beyond the scope of this study.

Functional annotation of DEG and DAP showed that almost 15% of identified changes are associated with phytohormone-related processes. Importantly, percentage of individual hormone category, aligns with changes in phytohormone content of *hvd14* (Figs. 2 and 3), showing close relationship between transcriptome/proteome and phytohormonal network. Both ABA and JA showed the most significant changes in 2-week-old, as well as 4-week-old plants (Supplementary Data 2). However, there is limited knowledge about the role of JA in branching, as well as its interactions with SL, we cannot exclude its involvement in the negative regulation of shoot architecture. Thus, the decreased levels of JA in 4-week-old *hvd14* might be linked with more 'bushy' phenotype and weaker plant responses to abiotic stress, which was proved in our previous study[32]. On the other hand, the SL and ABA relationship has been widely investigated, especially in terms of signalling pathway cross-talk during plants growth and development, as well as under environmental stress factors[61]. Our previous analysis showed that *hvd14* was insensitive to ABA during germination[62]. Moreover, we proposed that drought-sensitive phenotype of barley SL mutant might be caused by a disturbed ABA metabolism and/or signalling pathways. Thus, the most significant changes in ABA level and expression of ABA-related genes and encoding proteins highlight strong SL-ABA connection. Especially since BRC1 regulates the transcription of ABA-responsive regulators in axillary buds, including *ABA-RESPONSIVE ELEMENET BINDING FACTOR 3* (*ABF3*) and *ABA-INSENTIVIVE 5* (*ABI5*), by binding to the TCP motif present in their promoter sequences[63].

Our analysis also reveals three LOX genes, that were common between 2- and 4-week-old plants and were associated with phytohormone processes (Table 1). LOX catalyse oxygenation of free polyunsaturated fatty acids into oxylipins, a group of lipid compound, in which JA is included[64]. Literature data also indicate the involvement of LOX-like enzymes in the biosynthesis of ABA via cleavage of carotenoids to produce xanthoxin, which is rate-limiting step in the process[60]. LOX have been shown to be associated with biotic and abiotic stress responses in diverse plant species[65]. Genes encoding LOX are more expressed under stressful conditions, so elevated concentrations of plant defence hormones can trigger signal transduction, including SA, JA and ABA, leading to the plant's response to adverse environmental conditions[66–70]. However, here we showed that *hvd14* mutant presented lower content of JA, SA and ABA compared to WT, despite the increased expression of the genes encoding LOX during plants growth. Perhaps, the decreased content of these hormones, stimulates their biosynthesis as a feedback regulation, however the accumulation of ABA, JA and SA is blocked by unknown mechanisms. Additionally, the tissue used for transcriptome and proteome analysis was collected from the leaves, while hormone profiling involved the entire above-ground part of the plants.

SL-dependent TF involved in barley development

In well-studied model species like Arabidopsis or rice, the SL signalling pathway and its constituent proteins are extensively documented, from signal perception to repressor degradation. However, our understanding of downstream SL transcriptional responses remains basic. Investigating the transcriptome and proteome of *hvd14* and WT, we identify potential TF influencing SL signal transduction regarding barley development (Fig. 6). In total, 109 potential SL-related TF were identified among DEG and DAP in both 2- and 4-week-old WT and mutant plants, among which four Arabidopsis homologs (*AT3G18550*, *AT5G67060*, *AT1G64380*, *AT2G02820*) were already described as SL-responsive (Supplementary Data 7). One of the genes, *AT3G18550*, encodes a BRC1, the role of which in SL-dependent branching has been extensively demonstrated[3,5,11]. The second gene, *AT5G67060,* encodes HECATE 1 (HEC1) basic helix-loop-helix (bHLH) TF involved in the control of shoot meristem dynamics and gynoecium patterning by modulation of AUX and CK balance[71]. *AT1G64380* encodes an ETHYLENE RESPONSIVE FACTOR 61 (ERF61), which directly regulates the expression of nine genes involved in carotenoid biosynthesis, the precursor of SL or ABA[72]. Thus, the interaction between SL and ABA might occur at the biosynthesis level and be regulated by the feedback loop within SL signalling. Since the SL and ABA cross-talk has been widely documented under control and stress conditions, the ERF61 might be a good candidate for explaining the interaction between these hormones[61]. Another gene, *AT2G02820*, which was differentially expressed in the comparison of 2-week-old *hvd14* and Sebastian plants, encodes MYB DOMAIN PROTEIN 88 (MYB88) involved in a wide range of developmental processes, as well as plants response to abiotic stresses. It was shown that MYB88 and FOUR LIPS (FLP) control the guard cell differentiation and modulation of root architecture under drought conditions. In our previous analysis, we showed that *hvd14* under drought conditions presented a weaker response compared to WT, which was connected with lower leaf relative water content (RWC), impaired photosynthesis, disorganisation of chloroplast structure, slower closure of stomata, as well as altered stomatal density[62]. The impaired SL signalling in *hvd14* mutants could alter the activity of *MYB88*, thereby affecting the phenotype of mutant plants through reduced differentiation of guard cells. Additionally, *MYB88* is directly regulated by BRI1 ETHYLMETHANE SULFONATE SUPRESSOR1 (BES1), described as a co-regulator of Arabidopsis SL repressors.

Next, our bioinformatic approach reveals 33 potentially SL-responsive TF, which may regulate the expression of DEG and genes encoding DAP in 2- and 4-week-old plants (Fig. 4) (Supplementary Data 9). Functional enrichment analysis showed that this set of TF is mainly involved in hormone-associated processes, including response to hormone, hormone-mediated signalling pathways and response to abiotic stresses (Supplementary Fig. 1). These GO terms confirm that SL interacts with different phytohormonal pathways, reflected in disturbed hormone homeostasis in *hvd14* plants. Moreover, SL plays a key role in the activation of plants defence mechanisms under harsh environmental conditions, which might be explained by the alternation of ABA, JA and SA content in *hvd14*, as well as by the annotated function of identified TF.

Furthermore, a reanalysis of data presented by Wang et al., together with our bioinformatical approach, allows us to select 5 genes that encode TF, which may be involved in SL-signalling both in Arabidopsis and barley (Supplementary Data 11). The first one, *AT5G42520,* encodes BASIC PENTACYSTEINE6 (BPC6), which fulfils

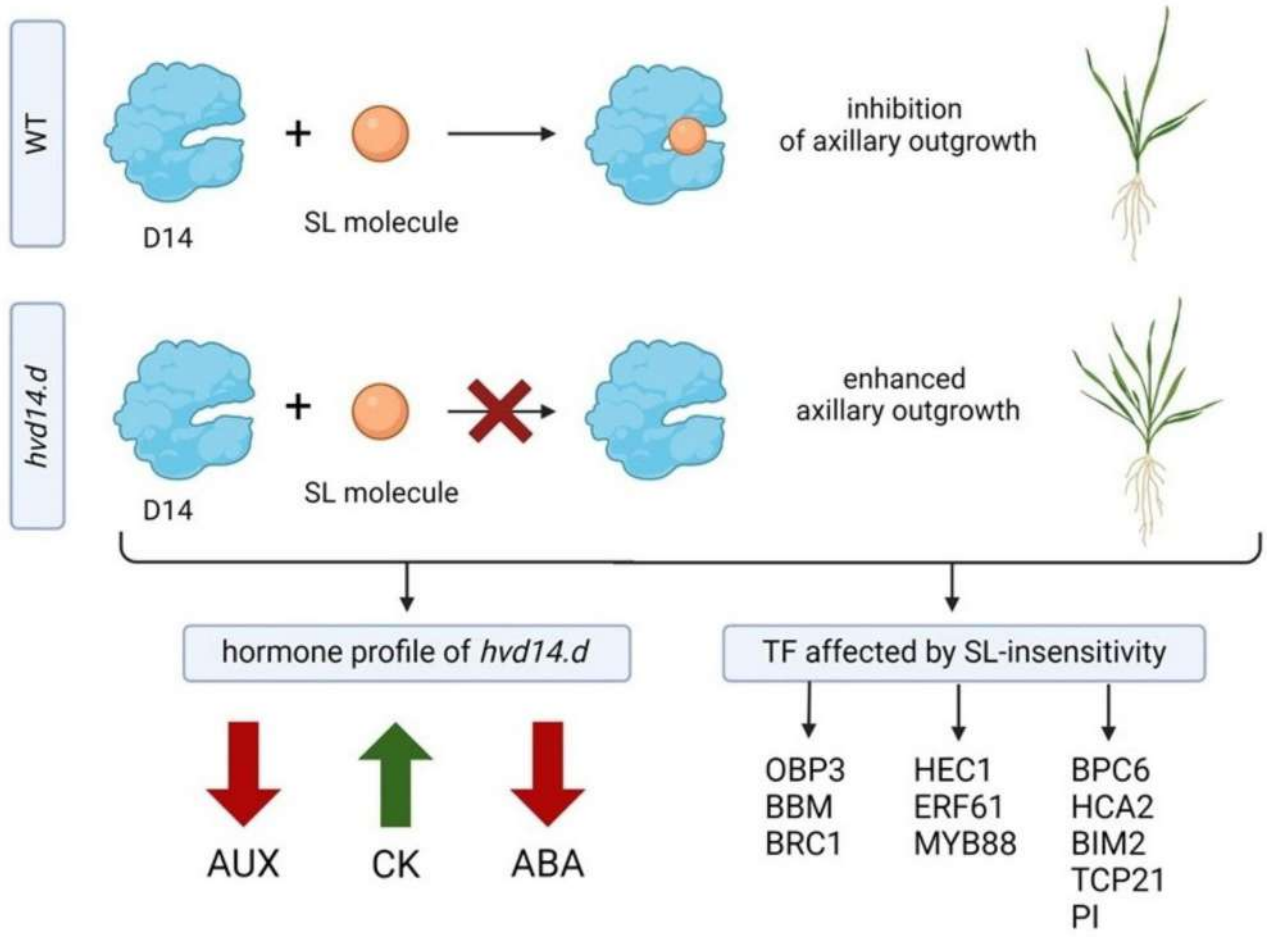


Fig. 6. Mutation in *HvD14* gene affects the shoot phenotype of barley due to altered hormone homeostasis and altered TF action. Mutation in *HvD14* gene leads to the loss of SL-molecule binding properties affecting the plants phenotype. Enhanced axillary growth of *hvd14* is connected with altered content of AUX, CK and ABA, as well as changes in TF activity. The illustration was created using BioRender (www.biorender.com).

indispensable functions in plant growth and development by coordinating a complex phytohormone network. BPC6 targets genes enriched for terms related to the response to ABA, AUX, JA, ET and CK, further supporting its role as a regulator of hormone signalling[73]. Indeed, genetic analysis showed that BPC6 promotes lateral root development by regulating ABSCISIC ACID INSENSITIVE 4 (ABI4) expression. Moreover, the roots abnormal phenotype of *bpc1 bpc2 bpc4 bpc6* plants was connected with diminished AUX transport due to reduced PIN1 accumulation, as well as invalid AUX response caused by down-regulation of *PLETHORA 1,2* (*PLT 1,2*) and *AUXIN RESPONSE FACTOR 7* (*ARF7*). The SL interaction between AUX and PINs proteins was already widely described, thus the *BPC6* is a strong candidate that may participate in SL-signalling. The second identified SL-related TF is *AT5G08330* encoding TCP DOMAIN PROTEIN 21 (TCP21). The TCP21 is an integral component of circadian clock, which together with TIMING OF CAB EXPRESSION 1 (TOC1), suppresses the transcription of CIRCADIAN AND CLOCK ASSOCIATED1 (CCA1), a master regulator of plants life cycle[74]. The circadian clock influences diverse developmental processes, especially the shaping of plants architecture[75]. It was shown that rice OsCCA1 positively regulated the expression of OsTB1, D14 and SPL14 to repress bud outgrowth[76]. Moreover, the downregulating and overexpressing OsCCA1 increases and reduces tiller numbers, respectively. Thus, the identified TCP21 might be another player involved in the tillering-circadian clock relation. Another gene, *AT5G62940,* encodes *HIGH CAMBIAL ACTIVITY 2 (HCA2),* which regulates interfascicular cambium formation and vascular tissue development[77]. Secondary growth is mediated by the vascular cambium, a stem cell-like tissue whose proliferating properties are regulated by the AUX and PIN proteins. Additionally, it was shown that SL-deficient mutants display a reduction in secondary growth, and local GR24 treatments stimulate cambium activity[78]. The fourth gene identified as a SL-related TF is *AT1G69010* encoding BES1-INTERACTING MYC-LIKE PROTEIN 2 (BIM2), which together with its homologs BIM1 and BIM3, interacts with BES1 known to activate the expression of BR-induced genes[79]. It was proved that BES1 also participate in SL signalling pathways, regulating the expression of downstream SL-related TF[80,81], highlighting the possible SL regulation of identified BIM2. The last gene identified as a SL-related TF is *AT5G20240* encoding homeotic protein PISTILLATA (PI), which were already described above.

Conclusion

A mutation in the *HvD14* gene encoding the receptor protein for SL contributes to semi-dwarf height and an increased number of tillers compared to the parent variety, Sebastian. The regulation of plant branching is influenced by environmental conditions and hormone balance, which affect transcriptomic and proteomic changes. Therefore, our SL-insensitive *hvd14* mutant was subjected to comparative analyses to understand the basis for the altered phenotype of these plants. Profiling the hormone content revealed significant differences in the levels of AUX, CK, and ABA, the role of which is well-known in shaping shoot architecture. It shows that the signaling pathway(s) regulating shoot branching operates as a fine-tuned system requiring a proper balance of hormone content. Moreover, mutation in *HvD14* resulted in a series of DEGs and DAPs, which allowed us to identify strong TF candidates that might be involved in SL signaling. The proposed SL-related TF have been previously indicated to interact with core SL-signaling proteins, as well as proteins primarily involved in AUX transport or ABA signaling, highlighting the complex interplay between these hormonal pathways in regulating plant growth and development. This study provides a comprehensive understanding of the genetic and molecular mechanisms underlying the altered *hvd14* phenotype, offering potential targets for further SL-related research.

Materials and methods

Plant material and growth conditions

The *hvd14* mutant carries a homozygous recessive mutation (G725A) in the gene encoding the SL receptor HvD14. This mutant was obtained using chemical mutagenesis after the double treatment of the parent cultivar Sebastian with sodium azide (NaN_3) and N-methyl-N-nitrosourea[31]. The mutant was double-backcrossed with Sebastian, and grains of both genotypes, Sebastian and *hvd14*, used in the presented studies were collected in this same year (2020).

The 15 grains of WT or mutant genotype, both sourced from the *Hor*TILLUS population[31] were sown in the boxes (400 × 140 × 175 mm) filled with soil containing a mixture of sandy loam and sand (7:2). Soil was supplied with a nutrient medium (per 1L: 34.3 g NH_4NO_3; 40.8 g KH_2PO_4; 10 g K_2SO_4; 61.5 g $MgSO_4{\cdot}7H_2O$; 0.05 g H_3BO_3; 0.03 g $CuSO_4{\cdot}5H_2O$; 0.01 g $MnSO_4{\cdot}H_2$; 0.81 g $FeCl_3{\cdot}6H_2O$) before sowing grains. The plants were grown in a growth chamber under a 16/8 h photoperiod at 20 °C. Analyses were performed on 14- and 28-old-day seedlings.

Phytohormone measurement

For phytohormone measurement, the whole shoot of seedlings was collected in four biological replicates, each containing four plants. Multiple phytohormone profiling by targeted metabolic analysis was applied to measure phytohormone content in barley tissue, as detailed described previously[82]. Three technical replicates were performed for each of two tissue sets for each genotype and time point. A paired Student's t-test was applied to check the statistically significant difference between samples.

Transcriptomic analysis

For RNA-seq analyses, plant tissue (2 cm long fragments of the second leaf located 3 cm below the leaf tip) was collected in four biological replicates, each containing fragments from 4 seedlings of both genotypes. cDNA libraries were prepared following Illumina TruSeq standard procedures and eventually sequenced in an Illumina NovaSeq6000 sequencer, producing 2 × 150 bp paired-end reads. The raw sequencing reads were analyzed using the FastQC software (v0.11.5, Cambridge, UK) to evaluate their quality. Adaptor sequences, empty reads, and low-quality reads ($Q < 30$ and length < 50 bp) were removed to generate high-quality clean reads. This trimming step was performed with the CLC Genomics Workbench software (v5.0, Qiagen, Vedbæk, Denmark). The clean reads were then aligned and quantified against the barley reference transcriptome using Kallisto (v0.43.0) with default parameters and 100 bootstrap iterations[83]. Differential gene expression analysis was conducted using the DESeq2 package[84]. Genes were considered differentially expressed if they exhibited a log2 fold change of ≥ 1 or ≤ -1 between conditions, with an adjusted p-value ≤ 0.01 following Benjamini–Hochberg correction.

Proteomic analysis

For proteome analysis, the whole shoot of seedlings was collected in four biological replicates, each containing four plants. The tissue was frozen in liquid nitrogen, ground mechanically and then dried using a freeze dryer equipped with a vacuum pump (LAB1ST; FDL1R-1A-220V; Irvine, CA 92,606, USA). The whole procedure requires three critical steps, including protein extraction, trypsin digestion and LC–MS analysis, which were described in detail previously[32]. Briefly, the protein extracts were prepared using an SDS-lysis buffer (4% SDS, 50 mM HEPES–KOH, pH 8.0) and clarified by centrifugation at 20.000 × g for 15 min at room temperature. Protein concentration was determined using a BCA assay (ThermoScientific, 23.225), and 500 µg of protein per sample was reduced with 10 mM DTT at 95 °C for 5 min, cooled, and alkylated with 30 mM iodoacetamide for 30 min in the dark. The reaction was quenched with 10 mM DTT. Samples were then prepared for trypsin digestion using a manual version of the R2-P1 protocol[85]. Peptides (1 µg) were analyzed using an Orbitrap Fusion Lumos Tribrid mass spectrometer, while raw mass-spec files were processed using MaxQuant software version 1.6.14[86]. Spectra were searched against a custom-made decoyed (reversed) version of the barley proteome from the r1 IBSC genome assembly (Phytozome genome ID: 462). Next, using Perseus version 1.6.14.0, reverse hits and contaminants were removed, the data was log-transformed and filtered based on valid quant values in at least 3 of 4 replicates per experimental group. Missing values were imputed from a normal distribution, and differentially abundant proteins were identified using a Benjamini–Hochberg corrected p-value threshold of < 0.05[87].

Gene ontology enrichment analysis

For GO enrichment analysis, the ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go77/) was used, with FDR cutoff set to 0.05 and the pathway dataset set to GO Biological Process. The tree map of GO Biological Processes were generated with REVIGO (http://revigo.irb.hr/) (the original tree map was modified using a graphic tool), with the resulting list set as medium (0.7) and $\log_{10}$ (size).

Identification of TF

For TF analysis, the protein sequences of DEG and DAP were obtained using the BioMart tool (https://sep2019-plants.ensembl.org/index.html) from the 'Hordeum vulgare genes (IBSC v2)' dataset. Next, with the PlantRegMap (https://plantregmap.gao-lab.org/) tool 'TF prediction', the TF among DEG and DAP were identified, parallel with their Arabidopsis homologs.

Promotor sequences analysis

For promotor sequences analysis, the 1500 bp before the codon START ('Flank Gene') of DEG and DAP were downloaded using the BioMart tool (https://sep2019-plants.ensembl.org/index.html) from 'Hordeum vulgare genes (IBSC v2)' dataset. Obtained files were used as input to identify potential regulatory interactions between TF and promoter sequences by PlantRegMap' Regulatory prediction' (https://plantregmap.gao-lab.org/), parallel with sorting out the TF which possess over-represented targets in the input gene set.

Data availability

All raw data used in this study can be found in the following repositories. Transcriptomic data: E-MTAB-12804: https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-12804; E-MTAB-12796: https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-12796. Proteomic data: PXD040828: https://www.ebi.ac.uk/pride/archive/projects/PXD040828

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Author contributions

Conceptualization: MMa; Investigation: MK, MMa, ADG, DM, RGU, ON, WB; Writing – original draft: MK, MMa; Writing – review & editing: MMa, ADG, DM, RGU; Funding acquisition: MMa, RGU, ADG, MK All authors read and approved the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

All procedures were conducted in accordance to the guidelines and legislation.

Additional information

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ROZDZIAŁ III.5

The cost of survival: mutation in a barley strigolactone repressor *HvD53A* impairs photosynthesis but increases drought tolerance

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The cost of survival: mutation in a barley Strigolactone repressor *HvD53A* impairs photosynthesis but increases drought tolerance

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Abstract

Strigolactones (SLs) are a class of plant hormones that play a crucial role in shaping plant architecture, significantly influencing plant adaptation to harsh environmental conditions. In this study, we examined the effects of a mutation in a component of the barley SL signalling pathway, the SL repressor *HvDWARF53A*, on plant growth and drought tolerance. We compared the results with those of a previously described barley mutant, which is highly tillered and drought-sensitive, carrying a mutation in the SL receptor gene HvDWARF14. The two mutants, *hvd14.d* and *hvd53a.f*, displayed contrasting phenotypes, including differences in plant height, tillering, and drought sensitivity. Under control conditions, ultrastructural analysis of *hvd53a.f* revealed smaller chloroplasts and fewer grana stacks, which may account for its reduced photosynthetic efficiency. Conversely, transcriptomic analysis linked the differentially expressed genes in *hvd53a.f* to antioxidation and stress responses, suggesting a potentially enhanced capacity to cope with drought. Further analysis revealed a strong connection between the SL signalling pathway and circadian clock components. Among these, CIRCADIAN CLOCK ASSOCIATED 1 emerged as a potential SL-responsive transcription factor (TF), possibly playing a key role in regulating tillering. Under drought conditions, *hvd53a.f* exhibited enhanced tolerance, as evidenced by higher relative water content, reduced chlorophyll degradation, and stable, albeit reduced, photosynthetic performance. Here, we identified the SL-related TF JUNGBRUNNEN 1 as a potential regulator of genes involved in water deficit response and antioxidation processes. Overall, the *hvd53a.f* mutation enhances drought tolerance while maintaining low, stable photosynthesis, highlighting *HvD53A* as a central node connecting SL signalling to stress resilience.

Keywords: barley • drought • DWARF14 • DWARF53 • Strigolactone • transcriptome

Introduction

Strigolactones (SLs) are a class of plant hormones initially discovered for their role as signalling molecules in interactions with root-parasitic plants (Cook et al. 1966). SLs, derived from carotenoids, act as a systemic signal that regulates diverse processes, including shoot branching, root architecture, and interactions with beneficial soil microbes (Bhoi et al. 2021). Plants harbouring mutations in genes encoding proteins involved in SL biosynthesis or SL signalling develop more lateral shoots than wild-type (WT), as observed in many model species including Arabidopsis (*Arabidopsis thaliana*), pea (*Pisum sativum*), rice (*Oryza sativa*), and maize (*Zea mays*) (Gomez-Roldan et al. 2008, Umehara et al. 2008, Braun et al. 2012, Guan et al. 2012, Liu et al. 2020). Although GR24 (a synthetic analogue of SLs) treatment can restore the phenotype of SL-deficient plants, it cannot rescue the phenotype of SL signalling mutants (Gomez-Roldan et al. 2008, Umehara et al. 2008). The SL signalling pathway constitutes a complex and finely regulated cascade initiated by the recognition and binding of the SL molecule to the receptor protein DWARF14 (D14), a member of the α/β hydrolase protein family (Hamiaux et al. 2012, Zhao et al. 2013). The functional SL receptor possesses a highly conserved catalytic triad formed by Ser96, His246, and Asp217, which is crucial for SL hydrolysis (Hamiaux et al. 2012). Crystallographic studies

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have shown that the D-ring of the SL molecule is trapped within the binding pocket of D14, thereby altering the conformational state (Zhao et al. 2013). This conformational change facilitates the interaction of the modified D14 receptor with key components of the SL signalling pathway complex.

The D14 receptor regulates the levels of bioactive SL molecules through ligand degradation (Seto et al. 2019). Binding of SL to D14 destabilizes the receptor, leading to its degradation via ubiquitination (Shabek et al. 2018). Recently, phosphorylation has been suggested to repress the ubiquitination and degradation of D14 in rice (Hu et al. 2024). The F-box protein that interacts with D14 following SL perception is a critical component of SL signal transduction. The F-box protein is a subunit of the SCF (SKP1-CULLIN-F-BOX) complex that targets transcriptional repressors for proteasomal degradation (Zhou et al. 2013). Degradation of SL repressors activates transcription factors (TFs) involved in SL signalling. Rice DWARF53 (D53) and its homologues in Arabidopsis, SUPPRESSOR OF MAX2-LIKE 6 (SMXL6), SMXL7, and SMXL8, are well-established negative regulators of SL signalling (Jiang et al. 2013, Zhou et al. 2013, Wang et al. 2015). Among the most crucial SL-dependent TFs is BRANCHED 1 (BRC1), a member of the TEOSINTE BRANCHED 1/CYCLOIDEA/PROLIFERATING CELL FACTOR 1 (TCP) family (Wang et al. 2019). In pea, *psbrc1* plants exhibit a 'bushy' phenotype that cannot be rescued by GR24 treatment (Braun et al. 2012). Additionally, the accumulation of BRC1 transcripts is significantly reduced in both SL-insensitive and SL-depleted plants. Moreover, the SL-dependent expression of BRC1 and its monocot homologue, *TEOSINE BRANCHED 1*, has been confirmed in numerous species, including Arabidopsis, rice, pea, wheat (*Triticum aestivum*), and maize (Aguilar-Martínez et al. 2007, Braun et al. 2012, Guan et al. 2012, Liu et al. 2017, Song et al. 2017).

SLs have also been recognized as critical hormonal regulators in plant adaptation to drought stress. Their role extends beyond shoot architecture regulation and includes modulation of stomatal conductance and interactions with other hormonal signalling pathways. In particular, the interplay between SLs and abscisic acid (ABA), —a central hormone in drought response, —has drawn significant attention in recent years. *In silico* analyses have revealed that cis-regulatory elements in the promoters of Arabidopsis and rice SL biosynthesis genes are associated with hormonal regulation (Marzec and Muszynska 2015). Most of these elements are linked to ABA-responsive factors, clearly emphasizing the crosstalk between SLs and ABA pathways. It has been shown that *d14* Arabidopsis and barley plants exhibit reduced drought resistance with slower ABA-mediated stomatal closure, altered stomatal density, and a thinner cuticle layer (Marzec et al. 2020, Li et al. 2020a, Daszkowska-Golec et al. 2023). Moreover, the transcription profile of ABA signalling genes, including *PYRABACTIN RESISTANCE 1-LIKE 4 (PYL4), SUCROSE NONFERMENTING 1 RELATED PROTEIN KINASES 2.1 (SnRK2.1)*, and *ABA INSENSITIVE 5* (*ABI5*), remained unchanged in the *hvd14* mutant under drought stress (Marzec et al. 2020). Similar results were noted in Arabidopsis plants harbouring mutations in the gene encoding the F-box protein, MORE AXILLARY GROWTH 2 (MAX2) (Bu et al. 2014, Ha et al. 2014). The max2 mutant was hypersensitive to drought and exhibited increased water loss compared to WT plants, due to a thinner cuticle layer, higher stomatal density, and inefficient stomatal closure caused by reduced responsiveness to ABA. However, analyses of mutants in genes encoding the F-box protein from the SCF complex (AtMAX2/OsDWARF3), related to SL signalling remain controversial, because these F-box proteins are also involved in karrikin (KAR) signal transduction, which has also been implicated in drought stress tolerance (Feng et al. 2022).

As the disruption of genes involved in core SL signalling results in drought hypersensitivity in plants, it is hypothesized that mutations in SL repressors would have the opposite effect due to the constitutive activation of the SL transduction pathway. In the Arabidopsis genome, three genes encode SL repressors, SMXL6, SMXL7, and SMXL8 (Wang et al. 2015, Soundappan et al. 2015). Characterization of single and double mutants under drought stress revealed that the knockout of one SL repressor gene did not affect plant survival rates compared to WT (Li et al. 2020b). However, mutations in two *SMXL* genes moderately increased drought resistance. Further studies have revealed that the triple mutant *smxl6,7,8* exhibits reduced cuticle permeability, increased anthocyanin biosynthesis, enhanced reactive oxygen species (ROS) detoxification capacity, and decreased water loss, all of which suggest enhanced drought survival.

Additionally, higher expression levels of *ABI5* and *SENESCENCE-ASSOCIATED GENE 29* (*SAG29*) were observed in *smxl6,7,8* mutants than in WT plants after 2 and 4 h of dehydration (Li et al. 2020b). Both these genes are widely used as markers for ABA responses, suggesting that the increased drought tolerance of *smxl6,7,8* plants is associated with ABA hypersensitivity. The interaction between SLs and ABA under drought stress has been linked to SL-mediated ABA sensitivity. It was demonstrated that treatment with GR245DS enhances plant drought tolerance by facilitating efficient stomatal closure, followed by an increase in the accumulation of the miR156 molecule in tomato leaves (Visentin et al. 2020). Moreover, this drought-induced upregulation of miR156 was absent in SL biosynthesis mutants compared to WT plants. Additionally, ABA-induced stomatal closure was more pronounced in miR156-overexpressing plants than in the WT (Visentin et al. 2020). These findings suggest that miR156 may serve as a key integrator of SL and ABA signalling pathways in the context of plant drought resistance.

In this study, we investigate how a loss-of-function mutation in a SL signalling repressor affects barley development and its response to drought. Using the *hvd53a.f* mutant, which carries a mutation in one of the barley SL repressors, *HvDWARF53A* (*HvD53A*), we examined physiological and transcriptomic responses under both control and drought conditions. To further dissect SL-dependent regulatory mechanisms, we also included the SL-insensitive *hvd14.d* mutant in our analyses. By integrating transcriptome profiling with phenotypic assessments, this work aims to uncover downstream targets of

SL signalling and provide new insights into how this pathway contributes to drought adaptation in monocot crops.

Results

Identification of *HvD53* genes and their alleles

The two barley orthologues of the rice SL repressor *OsD53* (Os11g0104300), HORVU.MOREX.r3.4HG0354980 (74% identity with the amino acid sequence of OsD53) and HORVU.MOREX.r3.5HG0466140 (71% identity with the amino acid sequence of OsD53), were named *HvD53A* and *HvD53B*, respectively. Both barley proteins contain the ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif (DLNxxP), involved in transcriptional repression in plants (Kagale and Rozwadowski 2011), and the RGKT motif (Supplementary Fig. S1) that is conserved in SL repressors identified in rice, Arabidopsis, and pea (Zhou et al. 2013, Soundappan et al. 2015, Kerr et al. 2021). Amino acid sequences of *HvD53A* and *HvD53B* exhibited a high level of identity (74%), and additionally, genes encoding both paralogues showed a high similarity in expression patterns (Supplementary Fig. S2). Within the 16 stages of barley development, both genes exhibited opposite expression patterns only in the senescing leaves (2-month-old plants) (Mascher et al. 2017, Li et al. 2023). The similarity in amino acid sequence and expression pattern indicates that *HvD53A* and *HvD53B* are paralogues that may encode functional SL repressors.

The TILLING strategy (Szurman-Zubrzycka et al. 2018) was applied to both *HvD53* genes, allowing for the identification of 16 new alleles (Supplementary Figs S3 and S4). Among *HvD53A* alleles, 10 affected the amino acid sequence of the protein. Moreover, four silent mutations and two mutations in the second intron were identified (Supplementary Fig. S3). In contrast, all mutations identified in the *HvD53B* sequence were localized in coding sequences, including 12 missense and four silent mutations (Supplementary Fig. S4). Homozygous M2 and M3 plants carrying missense mutations in each gene were phenotyped with respect to the number of tillers. Only plants carrying the *hvd53a.f* allele (T4001C, S664P) showed a statistically significant reduction in shoot branching compared to WT.

Phenotype of the *hvd53a.f* mutant and co-segregation analyses

To confirm co-segregation of *hvd53a.f* allele and the observed phenotype, the mutant plants were backcrossed with the barley cultivar 'Sebastian' and then crossed with the 'Golden Promise' cultivar. In both cases, the heterozygous F1 generation exhibited the WT phenotypes, whereas in the F2 generation, a 3:1 WT: mutant phenotype ratio was observed. Each F2 plant was genotyped and only homozygous *hvd53a.f* plants showed reduced shoot branching. In the Sebastian × *hvd53a.f* backcross, 293 F2 individuals were analysed, of which 84 contained only WT alleles, 134 were heterozygous, and 75 had only mutant alleles ($\chi 2H0 = 2.69$; $\chi 2P = 0.05$, $v = 2 = 5.99$). In the Golden Promise x *hvd53a.f* cross among 214 F2 plants: 51 had no mutation, 101 were heterozygous, and 62 were homozygous for the *hvd53a.f* mutation ($\chi 2H0 = 1.8$; $\chi 2P = 0.05$, $v = 2 = 5.99$). No recombinants with the mutant phenotype or WT allele of the *HvD53A* gene were found. This confirmed the assumption that the altered phenotype of the *hvd53a.f* mutant resulted from the identified recessive mutation.

All phenotypic analyses presented below were performed using F2 plants: WT (Sebastian or Golden Promise) and homozygous mutants named *hvd53a.f* (Seb) or *hvd53a.f* (GP). Statistically significant reductions in shoot branching were already visible in three-week-old *hvd53a.f* plants, compared to the WT genotypes, and continued until maturity (Fig. 1a–f). In mature plants, the *hvd53a.f* allele caused a reduction in branching by >30%, which was more strongly observed in the Golden Promise background (WT 15 ± 1.4 versus mutant 9 ± 1.4) than in the Sebastian background (WT 16 ± 1.4 versus mutant 11 ± 1.5) (Fig. 1f). The mutation in the SL repressor also increased mature plants' height by 14% in the case of Sebastian (WT 65.2 ± 2.73 cm, mutant 74.5 ± 3.44 cm) and 24% in the case of Golden Promise background (WT 55.9 ± 2.44 cm, mutant 69.7 ± 4.2 cm) (Fig. 1g). All homozygous plants carrying the *hvd53a.f* mutation were also noticeably pale green compared to WT. Hence, chlorophyll content was measured in the second leaf of four-week-old plants. These analyses showed a reduction in chlorophyll content by >40% in both genotypes (32.4 ± 3.2 a.u. versus 18.9 ± 1.78 a.u.—42% reduction in Sebastian background and 40.1 ± 3.62 a.u. versus 22.4 ± 3.57 a.u.—45% reduction in Golden Promise background) (Fig. 1h). Moreover, the *hvd53a.f* mutation delays flowering and harvesting. Plants were characterized by the appearance of the first visible awns (growth stage 49, according to the Zadoks decimal code) (Zadoks et al. 1974) to quantify the differences in flowering time. A mutation in the *HvD53A* gene delayed the appearance of the first visible awns by 19 and 12 days in the Sebastian and Golden Promise backgrounds, respectively (Fig. 1i). Lastly, the 3,3'-Diaminobenzidine (DAB) staining was performed to analyse the ROS scavenging efficiency of *hvd53a.f* and *hvd14.d* alleles (Supplementary Fig. S6). The lowest ROS accumulation was observed in *hvd53a.f* leaves under both control and drought conditions. In contrast, the highest ROS levels were observed in *hvd14.d* leaves under drought conditions. No other developmental differences, except for those mentioned above, were noted for *hvd53a.f* mutants.

A mutation in *HvD53A* affects chloroplast development and photosynthesis performance

To determine why *hvd53a.f* plants have decreased chlorophyll content, histological and ultrastructural analyses of leaf sections were performed. No difference in the number of chloroplasts was observed between *hvd53a.f* and WT plants (Fig. 2a). However, the total chloroplast area was smaller in the mutant (13.38 ± 2.494 μm2) than in the WT (14.57 ± 2.275 μm2) due to the reduced chloroplast length (5.49 ± 0.649 versus 6.73 ± 0.681 μm for *hvd53a.f* and WT, respectively) (Fig. 2b–d). The most significant differences were observed in the number of thylakoids and grana stacks, which were smaller

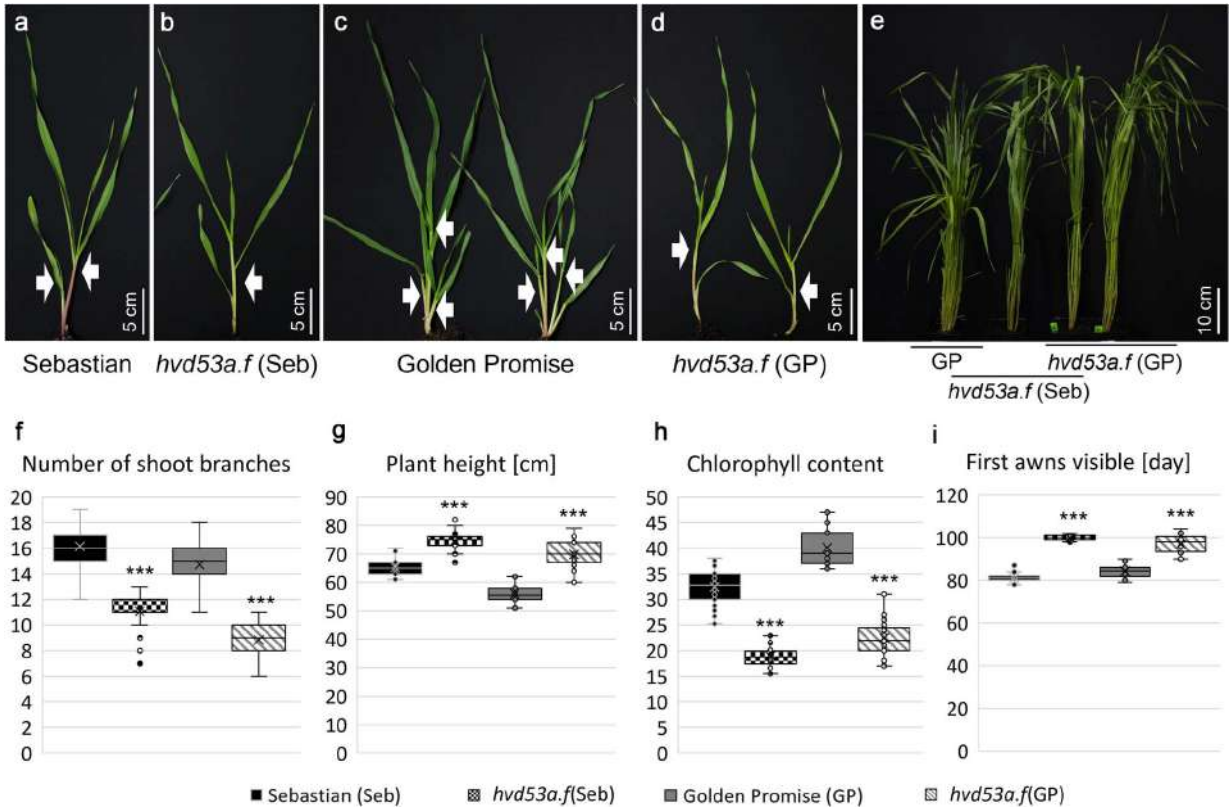

Figure 1. Phenotype of *hvd53a.f* plants. Differences in shoot branching among 3-week-old plants of (a) Sebastian, (b) *hvd53a.f* (Seb), (c) Golden Promise, (d) *hvd53a.f* (GP), and (e) 2-month-old plants. (f) The effect of the mutation in the *HvD53A* gene on shoot branching in mature plants, (g) the height of mature plants, (h) chlorophyll content in 4-week-old plants, and (i) flowering time are illustrated. Asterisks denote statistically significant differences between samples as per a paired Student's *t*-test (∗, ∗∗, and ∗∗∗ correspond to *P* values of .05, $P > .01$, $.01 > P > .001$, and $P < .001$, respectively).

in the mutant plants. Whereas the number of grana stacks was comparable between genotypes. The height of chloroplast stacks was significantly reduced in *hvd53a.f* (Fig. 2e—g).

Further analysis of photosynthetic performance revealed a highly reduced number of active reaction centres (RC/CS) in *hvd53a.f* (242 ± 20.7) compared to WT (916 ± 10.5). Also, the photosynthetic performance index (PIabs), a well-established indicator of photosynthesis efficiency, was significantly weaker in the mutant (0.36 ± 0.05 a.u.) than in WT (4.5 ± 0.17 a.u.). Poor photosynthetic efficiency is in line with the dissipation energy (DI/RC) observed in *hvd53a.f* (2.1 ± 0.14 versus 0.3 ± 0.007 a.u), which was substantially higher than in the WT (Fig. 3a—c). All the presented data highlight the drastically reduced photosynthetic performance linked to the inhibited chloroplast development caused by the mutation in the SL repressor.

Effect of the *hvd53a.f* mutation on the barley transcriptome

To get insight into the impact of *hvd53a.f* allele on gene expression profiles, transcriptomic experiments were conducted on four-week-old WT and mutant plants. In total, 4342 differentially expressed genes (DEGs) were identified in *hvd53a.f* leaves (2759 upregulated and 1583 downregulated) (Fig. 4) (Supplementary Data S1). Among the top 10 DEGs exhibiting the most significant changes in expression, four genes encoded members of the DEHYDRIN (DHN) protein family, with log2FC values ranging from 9.21 to 7.65 (Supplementary Data S1). Interestingly, the gene ontology (GO) enrichment analyses revealed three standard biological processes (BP) related to transcription found among up- and down-regulated genes in *hvd53a.f* (Fig. 4). Within up-regulated DEGs, processes related to oxylipin and glutathione metabolism, as well as protein phosphorylation, were identified, whereas down-regulated DEGs were primarily associated with various stress and stimulus responses (Fig. 4).

To identify genes whose expression is dependent on the SL signal transduction, comparative analyses were performed on the transcriptome of the barley mutant *hvd14.d*, which is insensitive to SLs (Marzec et al. 2020). The *hvd14.d* mutant was also identified using the TILLING strategy, and its insensitivity to SLs was attributed to a mutation in the *HvD14* receptor (Marzec et al. 2016). The *hvd14.d* and WT transcriptome comparison showed 5431 DEGs (3966 up- and 1465 down-regulated). GO

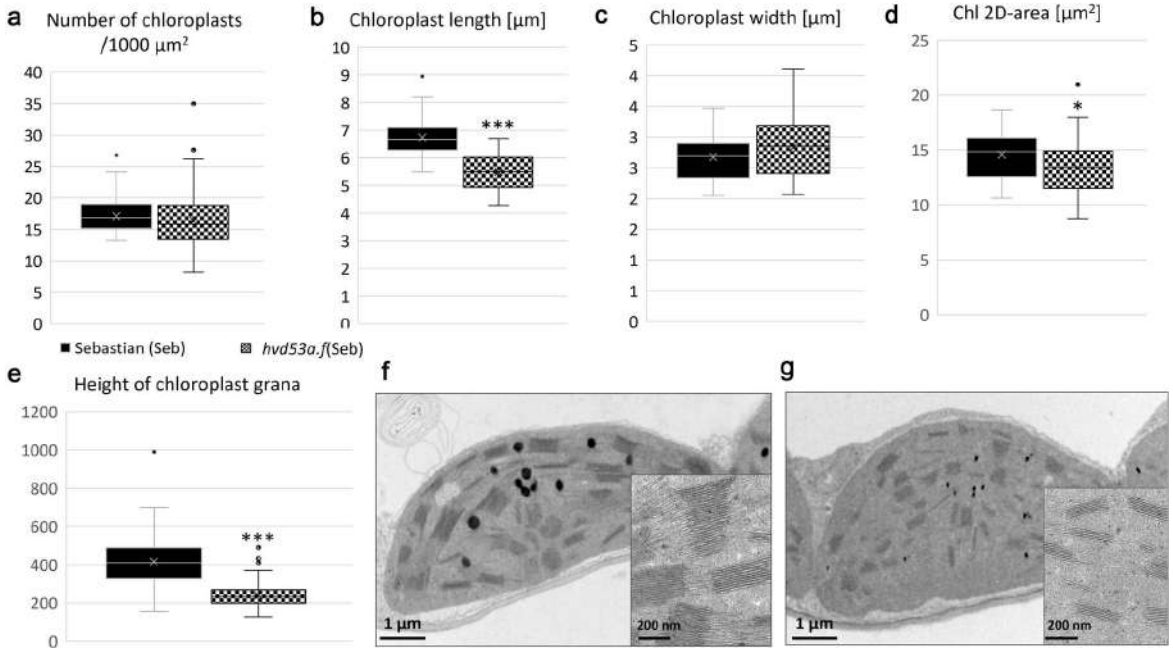

Figure 2. The impact of the *hvd53a.f* allele on chloroplast development. Impact of *hvd53a.f* allele on chloroplast development. Effect of mutation in *HvD53A* gene on (a) chloroplast density, (b) chloroplast length, (c) chloroplast width, (d) chloroplast area, and (e) chloroplast height. (f–g) Chloroplast and grana ultrastructure of WT and *hvd53a.f*. Asterisks indicate statistically significant differences between samples in a paired Student's *t*-test (∗, ∗∗, and ∗∗∗ correspond to *P*-values of $0.05 > P > .01$, $.01 > P > .001$, and $P < .001$, respectively).

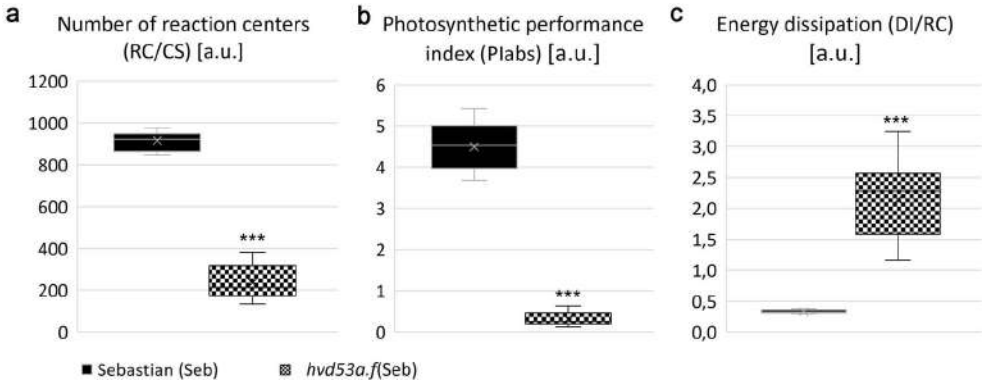

Figure 3. Impact of the *hvd53a.f* allele on photosynthetic performance. The effect of the mutation in the *HvD53A* gene on (a) the number of reaction centres per cross-section (RC/CS), (b) the photosynthetic performance index (Plabs), and (c) the dissipation energy per cross-section (DI/CS). Asterisks indicate statistically significant differences between samples in a paired Student's *t*-test (∗, ∗∗, and ∗∗∗ correspond to *P*-values of $.05 > P > .01$, $.01 > P > .001$, and $P < .001$, respectively).

analysis revealed that the up-regulated genes in *hvd14.d* were mainly associated with protein phosphorylation, while down-regulated genes were involved in RNA metabolism and transcription (Fig. 4) (Supplementary Data S1).

Comparison of DEG lists for *hvd53a.f* and *hvd14.d* revealed groups of genes common to both mutants (2005 up-regulated, 662 down-regulated), as well as those specific to *hvd53a.f* mutant (744 up-, 890 down-regulated), or specific for *hvd14.d* mutant (1930 up-, 793 down-regulated) (Supplementary Data S1). Interestingly, only 41 genes exhibited the opposite expression profile in both mutants (10 DEGs up-regulated in *hvd53a.f* and down-regulated in *hvd14.d*; 31 DEGs down-regulated in *hvd53a.f* and up-regulated in *hvd14.d*) (Table 1). In contrast, 61% of DEGs were identified for *hvd53a.f* (2667/4342) and 49% of *hvd14.d* DEGs (2667/5431) exhibited the same expression profile, indicating that mutations in the SL receptor (*HvD14*) and the SL repressor (*HvD53A*) destabilize the SL signalling pathway. The DEGs promoter sequences were analysed to discover the mechanisms responsible for transcriptome changes. These analyses revealed that TFs regulate the expression of each previously selected DEG set (Supplementary Data S2). As expected, a large number of TFs (22) were found to regulate the

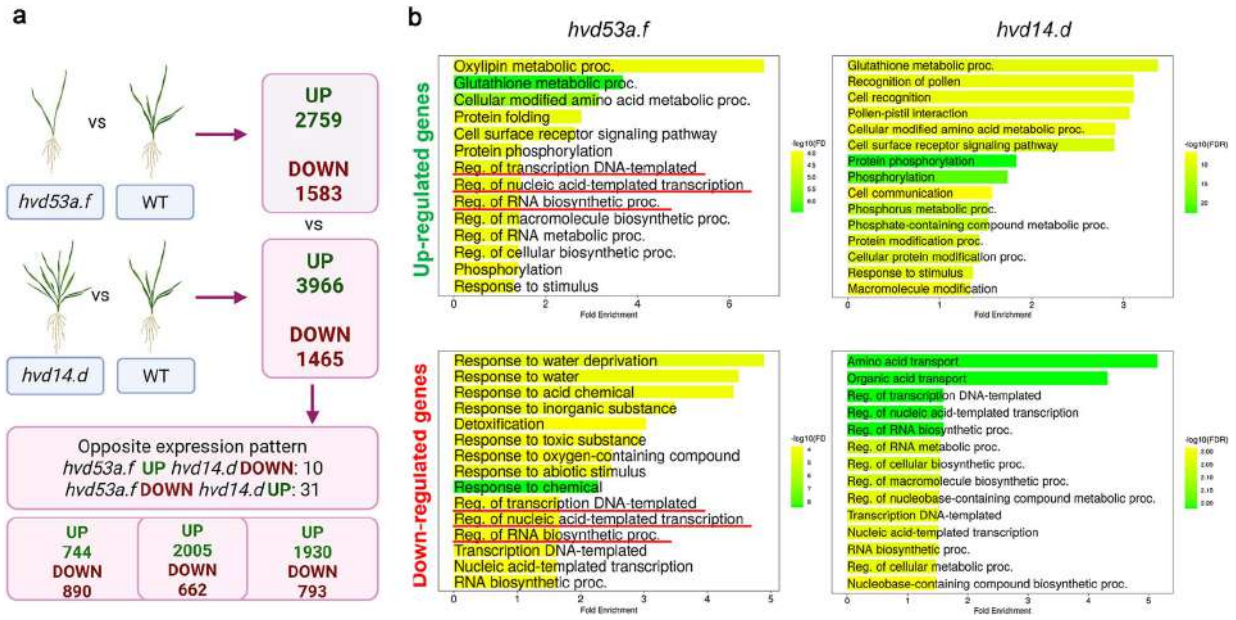

Figure 4. Overview of DEG identified in comparison of *hvd53a.f* SL repressor and *hvd14.d* SL receptor mutants versus WT Sebastian. (a) Summary of DEG specific/common for both mutants. (b) GO enrichment for up- and down-regulated genes identified for *hvd53a.f* and *hvd14.d*. The underline indicates the same processes identified in the pool of up- and down-regulated DEGs within the same genotype.

genes among all DEG sets, but TF specificity was still observed for common *hvd53a.f*/*hvd14.d* DEGs (19), for *hvd53a.f* (7) or *hvd14.d* (16) alone, and for DEGs with opposite expression profiles in both mutants (2) (Table 2). Interestingly, among TFs regulating the DEGs of *hvd53a.f* and *hvd14.d*, a key component of the circadian clock was identified. CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) (HORVU.MOREX.r2.7HG0579870) is an integral regulator of morning-phased gene expression, whose role is also associated with the regulation of the tillering process (Gong et al. 2022). To evaluate potential physical interactions between the SL signalling repressor D53A and CCA1, protein structure predictions were performed using AlphaFold2-based modelling. However, the inter-chain predicted TM-score (ipTM) and predicted TM-score (pTM) values, indicating the quality of the D53A—CCA1 complex, do not support direct interactions (Supplementary Data S3).

A mutation in *HvD53A* results in better adaptation to drought stress

SL mutants of many species are hypersensitive to water deficit, including the barley *hvd14.d* mutant (Marzec et al. 2020, Daszkowska-Golec et al. 2023). Thus, it was interesting to test the drought sensitivity of the SL repressor mutant, which exhibited a branching phenotype opposite to that of the SL receptor mutant. The obtained results indicate that *hvd53a.f* is less sensitive to drought than the WT and *hvd14.d*. The lowest reduction in dry mass was observed in the *hvd53a.f* mutant (29.9%), which was two- and three-fold smaller than that in the WT (67.6%) and *hvd14.d* (86.8%). The repressor mutant exhibited the highest relative water content (RWC) in the leaves after drought exposure (71.9%) among all the genotypes (WT, 58.8%; *hvd14.d*—38.5%). In addition, the reduction in chlorophyll content in response to drought in *hvd53a.f* (10.9%) was lower than in WT (19.4%) and *hvd14.d* (33.3%) (Fig. 5). Drought also affects the photosynthetic performance of the studied plants. Again, the smallest reduction of parameters among the studied genotypes was observed in *hvd53a.f*. At the same time, drought caused the most significant damage to the photosynthetic apparatus in *hvd14.d*. No statistically significant reduction in the number of reaction centres (RC/CS) was noted for the repressor mutant, which was not the case for the WT (9.5% reduction) and receptor mutant (26.7% reduction). Similarly, no differences in *hvd53a.f*, a 15% reduction in WT, and a 42.4% reduction in *hvd14.d* in response to drought were observed for the photosynthetic performance index (PIabs). Finally, the substantial increase of dissipation energy (DI/RC) was the most pronounced in *hvd14.d* (59.8%), whereas for WT, it was only 20%, and no statistically significant changes were noted for *hvd53a.f* (Supplementary Fig. S5).

The results suggest that the SL repressor mutant *hvd53a.f* shows improved drought tolerance compared to both the WT and the SL receptor mutant *hvd14.d*, exhibiting the least reduction in dry mass, higher RWC, and better photosynthetic performance under drought conditions. However, while *hvd53a.f* performed better than other genotypes, it should be emphasized that in absolute values, all described photosynthetic parameters of *hvd53a.f* were still the worst under both drought and control conditions.

Identification of SL-dependent transcriptomic responses to drought

To describe the transcriptome response to drought, plants exposed to stress were compared with those grown under

Table 1. DEG with opposite profile expression in *hvd53a.f* and *hvd14.d* mutants

#	HORVU ID (HORVU.MOREX.r2.)	Arabidopsis ID	Log2FC		Description
			hvd53a.f	*hvd14.d*	
1	7HG0556660	N/A	3.12	−8.91	N/A
2	5HG0351680	AT4G16730	2.95	−1.84	Terpene biosynthesis (AtTPS02)
3	7HG0620070	AT4G35160	2.38	−2.15	Melatonin synthesis (AtASMT)
4	7HG0600390	N/A	2.23	−1.47	N/A
5	2HG0090840	AT5G65400	2.07	−2.32	Alpha/beta-hydrolases superfamily protein.
6	7HG0604530	AT5G57550	2.01	−1.33	Cell wall remodelling via xyloglucan modification (AtXTH25)
7	7HG0620030	AT4G35160	1.73	−3.63	Melatonin synthesis (AtASMT)
8	UnG0628630	N/A	1.26	−1.12	N/A
9	6HG0456290	AT1G61680	1.15	−1.25	Biosynthesis of volatile terpenoids, S-linalool (AtTPS14)
10	1HG0020310	AT5G03170	1.10	−3.62	Mechanical properties of the plant's stem and cell wall (AtFLA11)
11	2HG0083580	AT5G07990	−5.44	2.86	Cytochrome P450 71A26
12	1HG0077320	AT1G72060	−4.18	2.19	Anthocyanin biosynthesis
13	6HG0523580	AT4G00350	−3.43	1.07	Multidrug and toxin efflux transporter (AtSNI1)
14	1HG0040410	AT5G62150	−2.96	1.22	Transporter belongs to the MATE (multidrug and toxin extrusion) efflux family
15	6HG0511990	N/A	−2.65	1.41	N/A
16	6HG0455680	AT3G16660	−2.55	2.13	N/A
17	3HG0219510	N/A	−2.53	1.33	N/A
18	6HG0469160	AT1G30260	−2.50	1.68	Response to cytokinin
19	2HG0091250	AT2G24960	−2.47	1.44	Cell differentiation, development, and responses to environment
20	4HG0278540	N/A	−2.27	1.43	N/A
21	6HG0498130	AT4G14740	−2.26	1.81	Regulate the localization of PIN1, auxin canalization protein (AtFL3)
22	1HG0044610	AT2G20750	−2.12	1.22	Expansin: loosening and extension of plant cell walls (AtEXPB1)
23	2HG0086370	AT5G66110	−2.10	3.34	Heavy metal binding and stress responses (AtHIPP27)
24	5HG0423630	AT4G34135	−2.07	1.33	Glucosylation of flavonols; in stress or defense responses (AtUGT73B2)
25	1HG0059270	AT5G59910	−1.96	1.03	Histone H2B (AtHTB4)
26	4HG0286320	AT3G19000	−1.91	1.19	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
27	7HG0559480	AT1G24470	−1.81	3.95	Synthesis of cuticular waxes and suberin precursors (AtKCR2)
28	7HG0601350	N/A	−1.81	1.05	N/A
29	2HG0157620	AT1G16510	−1.68	1.33	Cell expansion and auxin transport (AtSAUR41)
30	2HG0145740	AT3G48360	−1.43	1.39	Adapter of an E3 ubiquitin-protein ligase complex (CUL3-RBX1-BTB) (AtBT2)
31	6HG0521450	AT5G24560	−1.42	1.13	Putative F-box protein PP2-B12; member of the phloem protein family
32	5HG0367880	N/A	−1.35	1.08	N/A
33	6HG0513160	AT1G77760	−1.32	1.68	Converting nitrate into nitrite (AtNIA1)
34	5HG0424190	AT5G04885	−1.32	1.56	Xyloglucan metabolism (sugars and cell wall components) (AtBGLC3)
35	3HG0265830	AT3G57240	−1.29	1.92	Cell wall remodeling (AtBG3)
36	1HG0053720	N/A	−1.26	2.27	N/A
37	5HG0354930	AT3G03341	−1.23	1.63	Cold-regulated protein
38	6HG0490280	N/A	−1.18	1.62	N/A
39	3HG0226990	N/A	−1.12	1.44	N/A
40	1HG0014110	N/A	−1.11	1.77	N/A
41	1HG0021680	AT3G15630	−1.11	1.28	Active during pollen germination

control conditions. The lowest number of drought-induced DEGs was identified for *hvd53a.f* (5,043), whereas 8088 and 9909 DEGs in response to drought were found for WT and *hvd14.d*, respectively (Fig. 6a) (Supplementary Data S4). Among them, 1942 were specific for WT (968 up-regulated, 974 down-regulated), 711 were specific for *hvd53a.f* (400 up-, 311 down-regulated), and 3708 were specific for *hvd14.d* (1,484 up-, 2224 down-regulated). There are also 617 DEGs (240 up- and 377 down-regulated) common for both mutants; 30 genes up-regulated in WT and down-regulated in both mutants, as well as 137 genes with opposite expression profiles in *hvd53a.f* and *hvd14.d*. Finally, DEGs involved in barley response to drought, which are not SL-related were identified, including i.e. 2678 (1417 up- and 1261 down-regulated) genes exhibiting the same expression profile in all their genotypes during the drought response (Fig. 6b) (Supplementary Data S4).

Next, to identify SL-related TFs that modulate barley response to drought, we undertook further analyses focused on resolving (i) drought-induced DEGs common for all genotypes; (ii) drought-induced DEGs specific for each genotype; (iii) DEGs exhibited the same pattern in both mutants; (iv) DEGs with opposite expression profiles in *hvd53a.f* and *hvd14*, and (v) DEGs exhibited the same pattern in both mutants and opposite in WT (Supplementary Data S5). A comparison of the obtained lists reveals a lack of universal TFs for all DEG categories presented above. In contrast, the highest number

Table 2. List of exclusive TF potentially regularizing the expression of DEG specific or common for *hvd14.d* and *hvd53.f*, and presenting opposite expression pattern between *hvd14.d* and *hvd53.f*

#	Genotype	mloc_id	Best hit in Arabidopsis	Description
1	d14	MLOC_60958	AT2G02080	Indeterminate(ID)-domain 4
2		MLOC_75886	AT4G25480	Dehydration response element B1A
3		MLOC_10556	AT4G32730	Homeodomain-like protein
4		MLOC_58950	AT4G17980	NAC domain containing protein 71
5		MLOC_3926	AT1G26780	myb domain protein 117
6		MLOC_72007	AT1G25550	G2-like family protein
7		MLOC_72393	AT5G08330	TCP family protein
8		MLOC_10987	AT1G34370	C2H2 family protein
9		MLOC_78895	AT1G17950	MYB domain protein 52
10		MLOC_64795	AT4G30080	Auxin response factor 16
11		MLOC_14401	AT3G27785	MYB domain protein 118
12		MLOC_43537	AT2G20570	GBF's pro-rich region-interacting factor 1
13		MLOC_72275	AT5G08520	MYB family protein
14		MLOC_55345	AT1G19850	ARF family protein
15		MLOC_65400	AT5G39610	NAC domain containing protein 6
16		MLOC_52114	AT3G04030	G2-like family protein
1	d53	MLOC_74813	AT1G76890	Trihelix family protein
2		MLOC_60577	AT1G58100	TCP family protein
3		MLOC_78652	AT1G03840	C2H2 family protein
4		MLOC_17690	AT1G08320	bZIP family protein
5		MLOC_76196	AT5G59820	C2H2 family protein
6		MLOC_57518	AT1G55110	Indeterminate(ID)-domain 7
7		MLOC_75795	AT4G29230	NAC domain containing protein 75
1	Common	MLOC_5375	AT4G18960	MIKC_MADS family protein
2		MLOC_23250	AT1G67710	Response regulator 11
3		MLOC_53943	AT1G32240	G2-like family protein
4		MLOC_60074	AT2G01060	G2-like family protein
5		MLOC_65286	AT5G53950	NAC family protein
6		MLOC_14844	AT5G45580	G2-like family protein
7		MLOC_52944	AT2G45650	AGAMOUS-like 6
8		MLOC_37843	AT3G17730	NAC domain containing protein 57
9		MLOC_58026	AT1G69310	WRKY DNA-binding protein 57
10		MLOC_14619	AT2G27050	ETHYLENE-INSENSITIVE3-like 1
11		MLOC_71128	AT1G65910	NAC domain containing protein 28
12		MLOC_68284	AT3G18400	NAC domain containing protein 58
13		MLOC_14118	AT2G46830	CIRCADIAN CLOCK ASSOCIATED 1
14		MLOC_19175	AT4G10350	NAC domain containing protein 70
15		MLOC_24269	AT5G23280	TCP family protein
16		MLOC_36942	AT5G64060	NAC domain containing protein 103
17		MLOC_15014	AT1G73360	Homeodomain GLABROUS 11
18		MLOC_67851	AT2G38470	WRKY DNA-binding protein 33
19		MLOC_57142	AT1G51600	ZIM-LIKE 2
1	Opposite	MLOC_81350	AT2G23340	DREB and EAR motif protein 3
2		MLOC_38232	AT2G33860	ARF family protein

of unique TFs was identified as specific only for *hvd14.d* (19 TFs), while the remaining categories contained only one to five associated TFs (Supplementary Data S6). Furthermore, the most numerous lists of common TFs (21) characterized drought-induced DEGs across the first, second, and third categories, where almost 50% of these TFs belong to ETHYLENE RESPONSIVE FACTOR FAMILY (ERF) and are related to plant stress response. The analysis also revealed six TFs that are common across most of the categories, representing the first, second, third, and fourth. Among these TFs, two Arabidopsis homologues (AT2G43000 and AT2G40350) have been linked to the regulation of senescence and tolerance to various abiotic stresses, including drought. The barley homologue of AT2G43000 that encodes JUNGBRUNNEN 1 (JUB1), was further analysed using AlphaFold2 to assess its potential to form a protein complex with the SL repressor D53A. Here, both ipTM and pTM values do not indicate complex assembly (Supplementary Data S3). Furthermore, we compared the generated lists of TFs with previously identified TFs that regulate drought-induced DEGs (Daszkowska–Golec et al. 2023). Among the 27 TFs, 15 were common to our current analysis, highlighting them as likely players in the SL-regulated plant drought response (Supplementary Data S6).

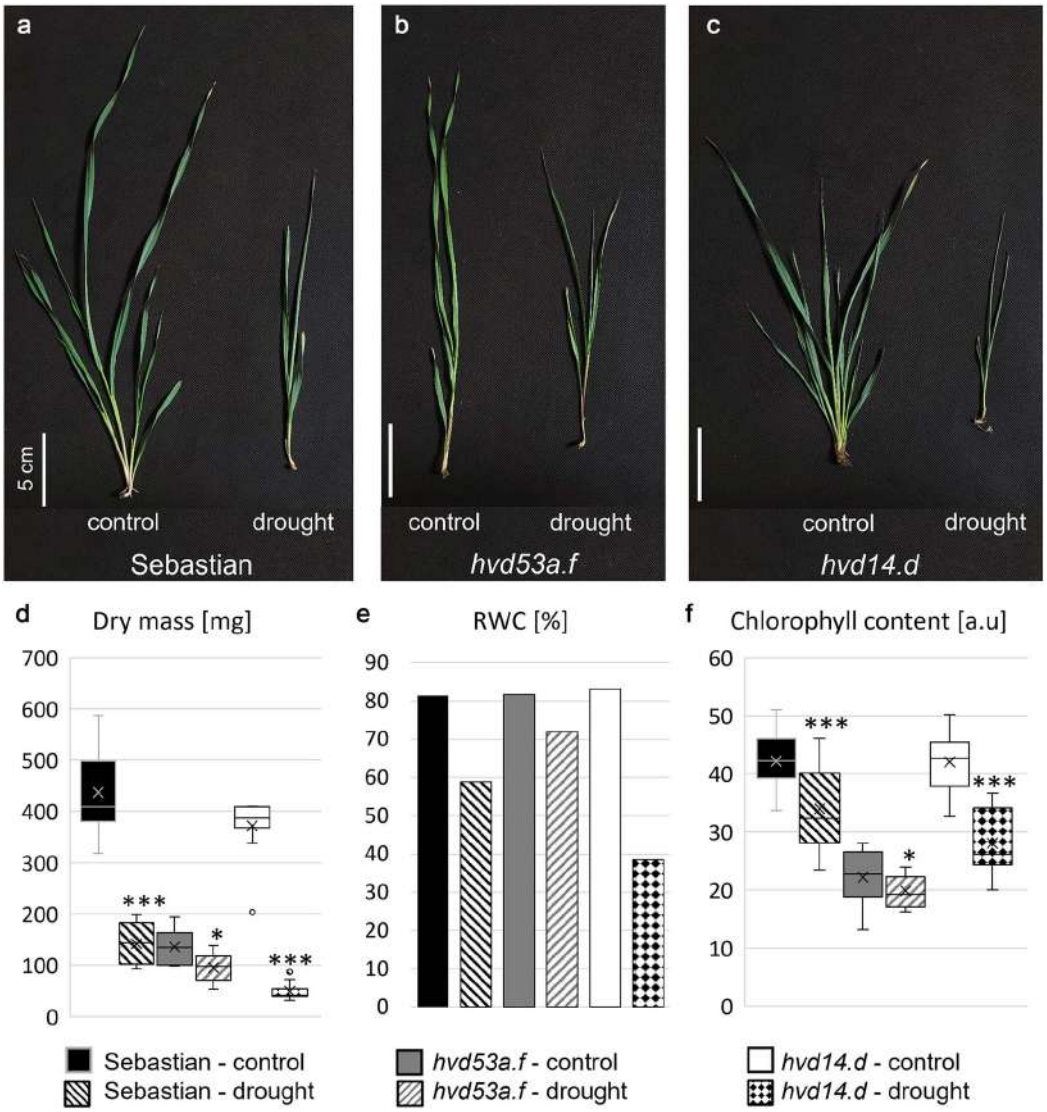

Figure 5. Drought response of SL barley mutants: The phenotypes of (a) WT Sebastian, (b) *hvd53a.f*, and (c) *hvd14.d* 4-week-old plants grown under control and drought conditions. The effects of drought on (d) dry mass, (e) RWC, and (f) chlorophyll content in the analysed genotypes are shown. Asterisks indicate statistically significant differences between samples in a paired Student's *t*-test (∗, ∗∗, and ∗∗∗ correspond to *P*-values of $.05 > P > .01$, $.01 > P > .001$, and $P < .001$, respectively).

Discussion

A mutation in barley Strigolactone repressor *HvD53A* affects shoot architecture and flowering time

The TILLING strategy allowed us to identify the first barley mutant of the SL repressor, *HvD53A*. Only homozygous plants displayed a reduced shoot-branching phenotype, indicating that *hvd53a.f* is a recessive allele. Phenotypic analyses revealed that the mutation in *HvD53A* resulted in a significant reduction in tiller number, increased plant height, and delayed flowering in both the Sebastian and Golden Promise backgrounds (Fig. 1). Similar observations were noted in the case of the Arabidopsis triple mutant *smxl6/7/8*, where T-DNA insertions caused a knockout mutation or truncation of the EAR motif, resulting in a dysfunctional protein (Wang et al. 2015). Moreover, the triple mutant *smxl6/7/8* completely restored the SL biosynthesis max3 phenotype and significantly decreased the secondary branch number.

On the other hand, rice mutants with a dominant mutation in OsD53 exhibited the opposite phenotype, characterized by an increased number of shoot branches. This is due to a gain-of-function mutation that prevents ubiquitination and degradation of OsD53 (Jiang et al. 2013, Zhou et al. 2013). Thus, the identified missense mutation in the barley SL repressor likely negatively affects the function of *HvD53A* that is hence no

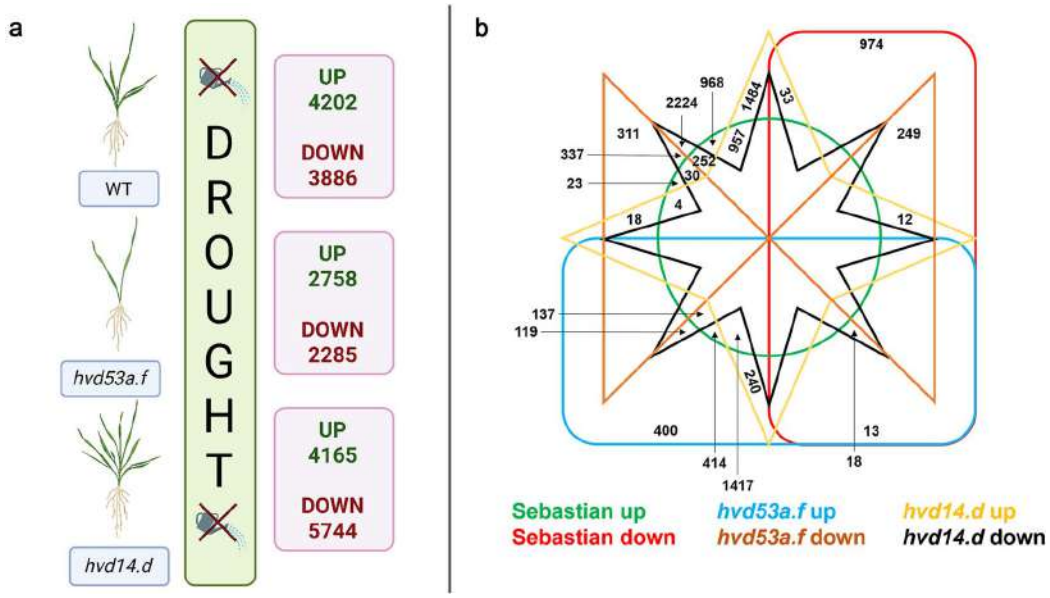

Figure 6. Overview of drought-induced transcriptome response in analysed genotypes. (a) DEG identified for each genotype. (b) Venn diagram for identified DEG.

longer capable of binding to SL-responsive genes or of interacting with other components of the SL signalling pathway. Besides architectural alterations, plants with an effective mutation in the *HvD53A* gene also exhibit delayed flowering and harvesting times.

To date, the role of SLs in the plant life cycle has been primarily associated with leaf senescence (Ueda and Kusaba 2015). However, recent studies have highlighted the potential role of SL in reproductive development. It was shown that both Arabidopsis SL biosynthesis and SL signalling mutants, *atmax3* and *atd14*, exhibit earlier flowering times compared to WT plants (Bai et al. 2024). Molecular analysis revealed that AtD14-mediated degradation of SMXL7 releases the AP2 family TF TARGET OF EAT 1 (TOE1) from its repressor complex. This enabled TOE1 to bind to and inhibit FLOWERING LOCUS T (FT) transcription, thereby repressing flowering. Moreover, the repressor triple mutant *smxl6/7/8* displayed delayed flowering, which was consistent with our findings regarding *hvd53a.f*. On the other hand, it was shown that HEADING DATE 3A (Hd3a), an FT orthologue from rice, might inhibit OsD53 degradation, resulting in attenuated SL signal transduction and leading to downregulation of OsTB1, thereby affecting tillering (Zheng et al. 2024). The oshd3a mutant exhibited decreased tillering and late flowering time, which might result from enhanced SL repressor degradation. Thus, both oshd3a and *hvd53a.f* exhibit a similar phenotype, likely due to disruptions in SL signalling, suggesting a regulatory interplay between Hd3a and D53 in modulating rice shoot architecture and development. The above Arabidopsis and rice studies suggest the possibility of a regulatory feedback loop between AtFT/OsHd3a and the SL signalling pathway, which together with our study, provides new insights into the hormonal control of plant flowering and tillering.

Disorganization of *hvd53a.f* chloroplast structure reduces photosynthesis performance

Barley d53a.f plants displayed a striking pale-green phenotype, with total chlorophyll content reduced by >40% compared to WT. To uncover the anatomical and ultrastructural basis of this chlorophyll depletion, we performed histological and electron-microscopic analyses on leaf sections. These studies revealed that *hvd53a.f* chloroplasts are smaller, contain fewer thylakoids and grana stacks, ultimately leading to diminished photosynthetic performance (Fig. 3). To date, alterations in the photosynthetic efficiency of SL-related mutants have primarily been studied under abiotic stress conditions (Ma et al. 2017, Ling et al. 2020, Marzec et al. 2020). Here, we demonstrate for the first time that a mutation in the SL signalling protein *HvD53A* drastically reduces photosynthetic performance owing to impaired chloroplast development under non-stressed conditions. The connections between chloroplast development and SLs remain poorly described.

The Arabidopsis and tomato (*Solanum lycopersicum*) microarray analysis showed that GR24 treatment induced the expression of light-harvesting genes (Mashiguchi et al. 2009, Mayzlish-Gati et al. 2010). In addition, sld14 plants exhibited reduced chlorophyll content, abnormal chloroplast structure, and reduced photosynthetic capacity (Li et al. 2022), which is reflected in our *hvd53a.f* mutant. However, due to the different roles of D14 and D53 in the SL signalling pathway, the observed consistent phenotypes remain intriguing. On the other hand, in the Arabidopsis d14 mutant, higher levels of chlorophylls were detected compared to WT (Li et al. 2020a). The divergent results regarding SL impact on chlorophyll accumulation in tomato and Arabidopsis indicate that the regulation of chlorophyll biosynthesis and degradation by the

SL signalling pathway is complex and may involve distinct downstream components or compensatory mechanisms in different plant systems. Given the central role of chloroplasts in energy-dependent reactions of oxygenic photosynthesis, impaired chloroplast development was observed in the *hvd53a.f* mutant, may exacerbate photosynthetic deficits by reducing the efficiency of light harvesting and electron transport. The smaller chloroplast size and fewer grana stacks in *hvd53a.f* mutant point to disrupted thylakoid membrane organization, which is crucial for photosystem II (PSII) activity and overall photochemical efficiency (Cackett et al. 2022). Our findings suggest a potential novel link between SL signalling and chloroplast development, proposing that *HvD53A* might act as a factor in optimizing photosynthetic efficiency. Further research is needed to elucidate the molecular mechanisms underlying this connection, including the identification of downstream targets of SL signalling that regulate plastid development and function.

Enhanced SL signalling due to mutated *HvD53A* affects the barley transcriptome, offering a better starting point for adaptation to stress

To better understand the molecular mechanisms underlying the differences between *hvd53a.f* and WT plants, a transcriptomic analysis was performed. This revealed a total of 4342 DEGs due to the mutation in the SL repressor, with 2759 and 1583 genes being up- and down-regulated, respectively (Fig. 4). The extensive number of identified DEGs suggests that SLs influence a wide range of BP, reflecting their critical role in plant growth and adaptation. GO enrichment analysis of the upregulated DEGs revealed that these genes were significantly associated with oxylipin metabolic processes and glutathione metabolism. Oxylipins, which are oxidation products derived from the catabolism of fatty acids, are known to be involved in plant stress responses (Creelman and Mulpuri 2002). However, numerous studies have highlighted their roles in flowering, leaf senescence, regulation of lateral root development, and ABA-independent and ABA-dependent stomatal closure (Vellosillo et al. 2007, Reinbothe et al. 2009, Montillet et al. 2013, Simeoni et al. 2022). Thus, enhanced activation of oxylipin metabolism may affect a wide range of processes. Furthermore, both oxylipins and glutathione play crucial roles in maintaining redox homeostasis within cells, the overactivation of which could contribute to the observed photosynthesis inefficiency in *hvd53a.f* plants (Herschbach et al. 2010, Noctor et al. 2012, Knieper et al. 2023).

Among the top 10 DEGs, four genes encoded DEHYDRIN (DHN) protein family (*HvDHN1–4*) proteins with log2FC values ranging from 9.21 to 7.65 (Supplementary Data S1). Dehydrins are crucial for membrane stabilization, ROS detoxification, and water retention, making them essential for stress tolerance, adaptation, and survival of plants exposed to challenging environmental conditions (Riyazuddin et al. 2022). Previous studies have shown that the expression patterns of all 13 dehydrin genes in barley (*HvDHN1–13*) vary widely under mild and severe drought conditions, with fold changes ranging from 0.4 to over 5500 (Abedini et al. 2017). This suggests that not all dehydrin genes are exclusively drought-induced and may play distinct roles in plant growth and development. Nevertheless, the increased expression of *HvDHN1–4* in the *hvd53a.f* mutant, as well as up-regulated metabolism of oxylipins and glutathione, may enhance its capacity to cope with drought stress, potentially offering a better starting point for stress adaptation. Moreover, DAB staining revealed the highest antioxidation efficiency of *hvd53a.f* mutant compared to WT and *hvd14.d*, further supporting this hypothesis (Supplementary Fig. S6). However, the down-regulated DEGs mostly mapped to the response to water deprivation and response to stimuli, implying that *hvd53a.f* plants might exhibit diminished stress responsiveness—a conclusion that conflicts with our observations. Perhaps, under control conditions, these genes are already less active in *hvd53a.f* plants, suggesting a shift in the overall stress response strategy. Rather than relying on these down-regulated genes, the mutant may activate alternative protective mechanisms, such as enhanced dehydrin expression, to improve drought resistance.

Alternatively, *hvd14.d*, which is insensitive to SL, exhibited the opposite phenotype to *hvd53a.f* (Marzec et al. 2016, 2020). A comparative analysis between both genotypes was performed to select genes and TFs, whose expression is dependent on SL signal transduction (Supplementary Data S1 and S2). The comparison revealed groups of genes that are common or specific for both *hvd53a.f* and *hvd14.d*. Each group consists of a large number of genes, which suggests that alterations in the SL signalling pathway have a broad impact on gene expression regulation, potentially leading to distinct phenotypes and adaptive strategies in response to environmental conditions. Of note, only 41 genes exhibited opposite expression profiles in both mutants (Table 1). We believe this limited number of oppositely regulated genes reflects the complex nature of SL signalling. In addition, our analysis suggests that barley likely has two functional SL repressors, which we named D53A and D53B, suggesting some redundancy. It is also possible that these genes are expressed in different tissues and/or at different times, which adds another layer of complexity. Moreover, the contrasting phenotypes might result from differences in gene expression levels rather than oppositely regulated genes, primarily since most of those 41 genes encode enzymes involved in metabolic processes.

Altered SL signalling of *hvd14.d* and *hvd53a.f* reveals an interaction between SLs and the circadian clock

To reveal the molecular background of contrasting phenotypes between *hvd14.d* and *hvd53a.f*, we performed a bioinformatic identification of SL-related TFs that might be responsible for transcriptomic changes in both mutants (Table 2). Among the 19 TFs that potentially regulate the expression of DEGs common to *hvd53a.f* and *hvd14.d*, CCA1 was identified (HORVU.MOREX.r2.7HG0579870). CCA1 protein plays a

key role as a regulator of the plant life cycle, controlling daily stomata opening, spike development, and shaping of shoot architecture (Hassidim et al. 2017, Wang et al. 2020, Gong et al. 2022). A study on rice revealed that *OsCCA1* directly promotes the expression of *OsTB1*, *OsD14* and *OsD10* (SL biosynthesis gene), thereby inhibiting bud outgrowth. Additionally, knockout or overexpression of *OsCCA1* resulted in an increased or decreased number of tillers, respectively (Wang et al. 2020). Thus, the altered activity of *HvCCA1* may explain the phenotypes of *hvd53a.f* and *hvd14.d*. Overexpression of maize (*Z. mays*) *ZmCCA1* led to reduced chlorophyll content, a trait also observed in *hvd53a.f* plants (Ko et al. 2016). Among the 16 TFs potentially regulating the expression of DEGs specific only for *hvd14.d*, TCP21 was selected (Table 2). TCP21 is a crucial player in the daily rhythm of plants that promotes the expression of evening-phased genes by repressing CCA1 activity. The dimerization of TCP21 with TIMING OF CAB EXPRESSION 1 (TOC1) prevents its binding to the *CCA1* promoter (Pruneda-Paz et al. 2009, Danisman 2016). Moreover, TCP21 was identified as a potential SL-responsive TFs in our previous bioinformatic analysis (Korek et al. 2025). Thus, the interactions between SLs and circadian clock regulation were strongly highlighted. To verify whether CCA1 could act as an upstream SL-related TF directly interacting with the SL repressor D53A, we used AlphaFold-Multimer to model the D53A–CCA1 complex. The resulting ipTM score was 0.2, suggesting that a stable direct interaction is unlikely. However, this result does not entirely exclude the possibility of interaction, as post-translational modifications such as phosphorylation could influence protein–protein binding (Friso and van Wijk 2015, Millar et al. 2019). Nevertheless, CCA1 may participate in the SL signalling pathway regulating shoot architecture through indirect mechanisms or alternative regulatory partners (Fig. 7a).

Only seven TFs were identified that potentially regulate DEGs specific for *hvd53a.f*. We found it interesting to recognize TCP8 as another TF from the TCP family that might be SL-responsive. TCP8 repressed the expression of FLOWERING LOCUS C (FLC), a central floral repressor. Overexpression of AtTCP8 results in delayed flowering through an FLC-dependent pathway (Wang et al. 2019). Moreover, analysis has revealed that FLC regulates flowering time by binding to and repressing FT expression (Deng et al. 2011). It was mentioned above that FT expression is also regulated by SMXL7-mediated degradation (Bai et al. 2024). Thus, SLs might coordinate holistic flowering timing by activating different pathways that regulate common genes involved in flowering.

SL-related *hvd53a.f* and *hvd14.d* mutants display contrasting phenotypes under drought

In addition to shaping the root and shoot architecture, SLs are also involved in the adaptation of plants to various abiotic stresses (Alvi et al. 2022). Consequently, SL-deficient or SL-insensitive mutants, including the barley *hvd14.d* mutant, display increased sensitivity to water scarcity (Marzec et al. 2020, Daszkowska-Golec et al. 2023). Therefore, assessing the drought response of the SL repressor mutant, which shows a contrasting branching pattern compared to that of the SL receptor mutant, is of particular interest. This is all the more true, since reduced tillering has been associated with water-saving and enhanced yield in water-limited conditions (Hammer et al. 2023).

Drought stress greatly influences light-dependent photosynthesis reactions. These reactions occur within thylakoid membranes, where chlorophyll captures light energy to produce ATP and NADPH through photosystem II and photosystem I, respectively (Chauhan et al. 2023). Since *hvd53a.f* plants possess reduced chlorophyll content and impaired photosynthetic efficiency under non-stressed conditions, we tested whether drought stress would exacerbate this issue. Various measurements, including dry mass, RWC, chlorophyll content, photosynthesis performance index, energy dissipation (DI/RC), number of reaction centres, as well as DAB staining, indicated that *hvd53a.f* was less sensitive to drought than both *hvd14.d* and WT (Fig. 5, Supplementary Fig. S6). However, it is essential to note that the absolute values of the measurements showing the photosynthesis efficiency for *hvd53a.f* were the lowest, compared to all genotypes. This suggests that while non-tillered *hvd53a.f* plants exhibit greater drought tolerance, their ability to maintain photosynthetic efficiency is inherently lower, possibly because of trade-offs that prioritize drought resilience over optimal photosynthetic performance (Fig. 8). Following this, several hypotheses might explain the interactions between tillering, photosynthesis, and drought response. Reduced photosynthesis may affect the levels of available photoassimilates, thereby limiting plant growth and ultimately reducing water consumption. Moreover, decreased photosynthetic efficiency might help mitigate damage from excessive ROS production and might conserve water, which would otherwise be required as an electron donor in photosynthesis. These mechanisms may enable plants to adapt to water scarcity.

Significantly higher drought tolerance was also observed in the Arabidopsis triple SL repressor mutant *smxl6/7/8* (Li et al. 2020b, Feng et al. 2022). An increased survival rate was associated with reduced cuticle permeability, enhanced anthocyanin biosynthesis, increased ROS detoxification capacity, decreased water loss, and increased sensitivity to ABA. Unfortunately, photosynthetic efficiency was not included in the analysis. Contrasting observations regarding *hvd14.d* have been described in our previous study, where mutant plants exhibited a hypersensitivity to drought phenotype. This is characterized by lower RWC, impaired photosynthesis, disorganized chloroplast structure, and altered stomatal closure and density (Marzec et al. 2020). The drought-sensitive phenotype of SL-depleted and SL-insensitive plants has been linked to reduced sensitivity to ABA in various species (Ha et al. 2014, Visentin et al. 2016, Haider et al. 2018, Marzec et al. 2020). Recently, *smxl6/7/8* have been suggested to directly bind to *SnRK2.3* thereby repressing its transcription (Feng et al. 2022). SnRK2 proteins are positive regulators of ABA signalling, leading to the phosphorylation of downstream ABA-related TFs. The Arabidopsis triple mutant *smxl6/7/8* was hypersensitive to ABA during the seed germination assay, whereas the mutation of *SnRK2.2/2.3* significantly

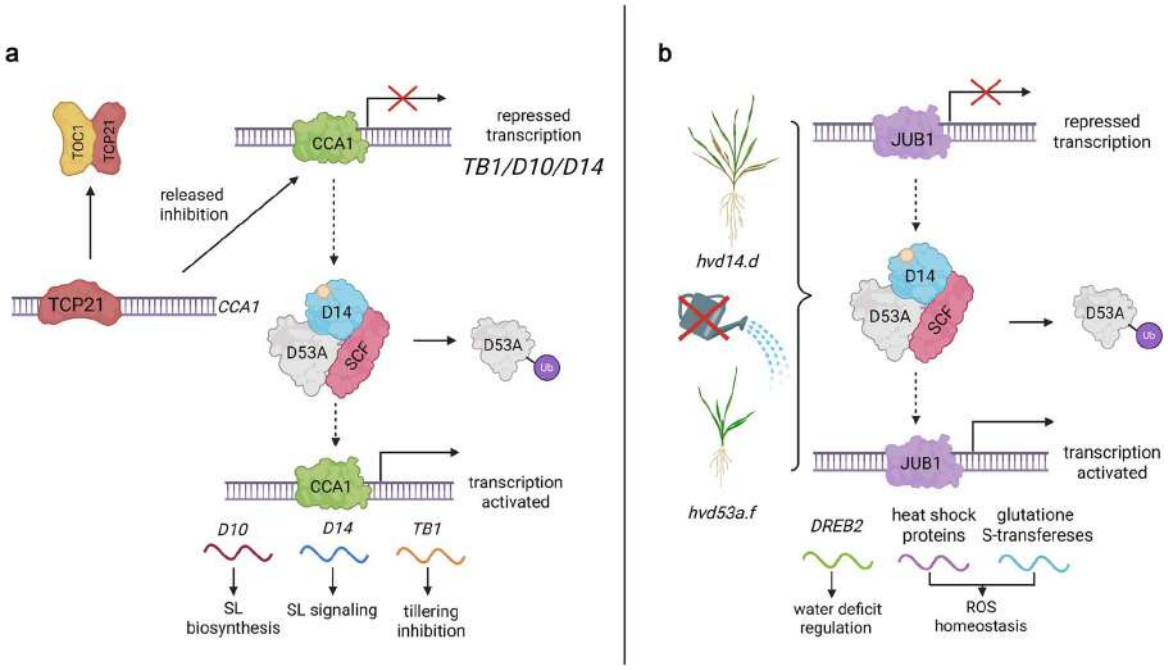

Figure 7. Proposed SL-related mechanisms explaining the phenotypic differences between *hvd14.d* and *hvd53a.f* under non-stress (a) and drought conditions (b). (a) CCA1 directly inhibits the expression of TB1, D14, and D10 by binding to their promoters. Upon recognition of SL molecules, the D14 receptor undergoes a conformational change, enabling its interaction with the F-box protein of the SKP1-CULLIN-F-box (SCF) complex. This leads to ubiquitination and subsequent degradation of the repressor protein by the 26S proteasome, releasing CCA1 via indirect mechanisms or alternative regulatory partners and allowing the transcription of D10, D14, and TB1. TCP21, in turn, inhibits CCA1 transcription, however, its dimerization with TOC1 prevents its binding to the CCA1 promoter, ensuring the presence of functional CCA1 protein. (b) SL signalling complex assembly affects the activity of JUB1, which controls the transcription of genes involved in ROS homeostasis and water deficit regulation. D53A—DWARF53A, CCA1—CIRCADIAN CLOCK ASSOCIATED1, TOC1—TIMING OF CAB EXPRESSION1, TCP21—TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR21, TB1—TEOSINE BRANCHED1, D10—DWARF10, D14—DWARF14, JUB1—JUNGBRUNNEN1, DREB2—DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2; dashed line—indicates proposed or indirect interactions that have not been experimentally validated, inferred from *in silico* predictions and literature-based assumptions.

suppressed this response. Thus, the enhanced drought tolerance of *hvd53a.f* plants may be associated with stronger activation of the ABA signalling pathway. The altered function of the *HvD53A* protein may lead to elevated SnRK2 transcription, resulting in the upregulation of drought-response genes.

SL-dependent regulation of drought in *hvd14.d* and *hvd53a.f*

Transcriptome analysis highlighted distinct drought response mechanisms among the tested genotypes, with *hvd53a.f* exhibiting the fewest drought-induced DEGs, suggesting a more efficient or preadapted response to water stress (Supplementary Data S4). In contrast, *hvd14.d* displayed the highest number of DEGs, indicating a greater level of transcriptional reprogramming, likely owing to its increased drought sensitivity. Among the 137 genes with opposite expression patterns, we identified *HORVU.MOREX.r2.6HG0458250* that encodes plasma membrane aquaporin (PIP) and shows enhanced and reduced expression in *hvd53a.f* and *hvd14.d*, respectively. Aquaporins facilitate the passive transport of water and other molecules, including ROS, and their expression in barley is affected by drought conditions (Kurowska et al. 2019). Thus, the presented expression pattern of the aquaporin gene in the tested genotypes might be related to measurements of RWC, as well as to the level of sensitivity to drought conditions.

Further, to reveal SL-dependent regulation of drought adaptation, we performed a bioinformatic identification of SL-related TFs, which might explain contrasting drought-sensitive and drought-resistant phenotypes of *hvd14.d* and *hvd53a.f*, respectively. This comparative approach allowed us to select TFs that might be specific or universal for the analysed genotypes (Supplementary Data S5). We identified Arabidopsis homologues of each TF to obtain a broader understanding of their potential functions and regulatory roles in the drought stress response. Notably, no single TF was universally present across all the DEG categories. However, we identified two TFs (Arabidopsis homologues AT2G43000 and AT2G40350) that regulate the highest number of DEGs across distinctive groups, linked to senescence and abiotic stress tolerance (Supplementary Data S6). Both of these processes

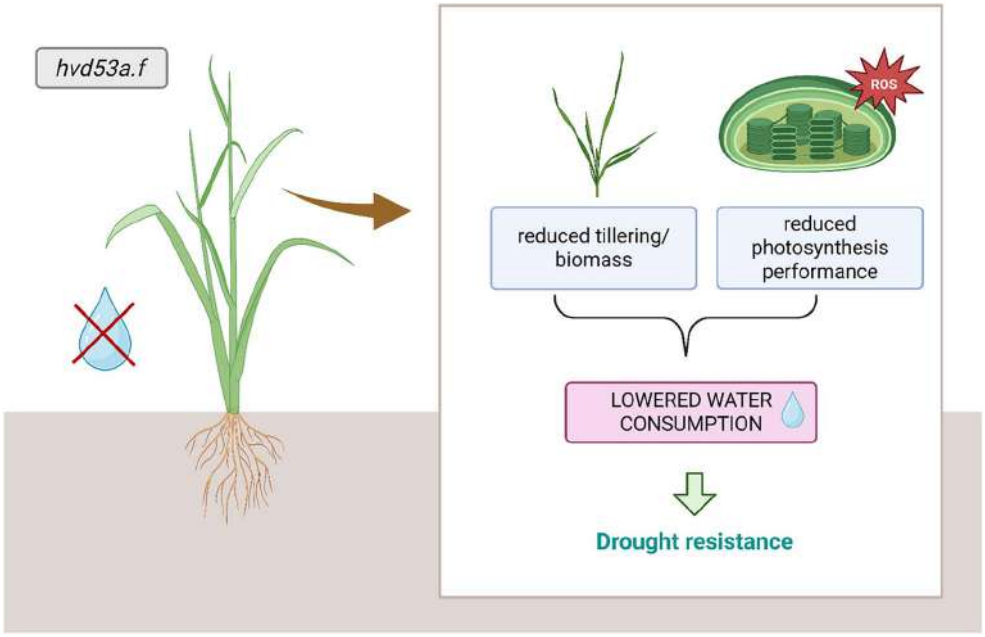

Figure 8. A mutation in *HvD53A* improves drought resistance at the cost of lowering photosynthesis efficiency. The mutation of the *HvD53A* gene results in decreased total biomass, primarily due to a lower number of tillers and diminished photosynthetic performance. Impaired photosynthesis results in a reduction in the accumulation of photoassimilates, thereby limiting plant growth and concurrently reducing water use. Furthermore, decreased photosynthetic activity may alleviate oxidative stress by minimizing the overproduction of ROS and contribute to water conservation, as water serves as an essential electron donor in the photosynthetic process. Collectively, these physiological adjustments may facilitate plant adaptation to water-deficient environments. This figure was created with BioRender.com.

are known to be modulated by SL pathways (Bu et al. 2014, Ueda and Kusaba 2015, Li et al. 2020b, Daszkowska-Golec et al. 2023). Overexpression of AT2G43000 encoding H2O2-induced JUNGBRUNNEN 1 (JUB1) protein strongly delays senescence and increases drought stress tolerance, whereas *atjub1* presents the opposite phenotype (Ebrahimian-Motlagh et al. 2017). Moreover, JUB1 regulates the expression of several ROS-responsive genes, including heat shock protein and glutathione S-transferase genes that are crucial for maintaining cellular redox homeostasis and enhancing stress tolerance (Wu et al. 2012). This regulation helps mitigate oxidative damage under drought conditions, thereby improving plant survival and adaptation, which we observed upon DAB staining (Supplementary Fig. S6). Thus, ROS-scavenging mechanisms might be part of an SL-dependent defence response that is disrupted in *hvd14.d*, leading to enhanced drought sensitivity, while its promotion in *hvd53a.f* contributes to drought resilience (Marzec et al. 2020) (Fig. 7b). Similarly to the previous analysis, we tested *in silico* the possibility of an interaction between the barley homologue of JUB1 and the D53A SL repressor. AlphaFold2 modelling yielded an ipTM score of 0.48, suggesting that although a weak interaction cannot be entirely excluded, the structural confidence is insufficient to support a direct and stable binding D53A and JUB1 under physiological conditions (Supplementary Data S3). However, this result does not exclude the potential involvement of JUB1 in SL signalling, especially considering our transcriptomic and promoter analyses, which consistently point to its SL-dependent regulation and possible upstream role. It cannot be excluded that D53A may influence JUB1-dependent gene expression through intermediary proteins, such as co-repressors, chromatin remodelers, or transcriptional cofactors that modulate JUB1 activity. Another possibility is that D53A affects the stability, localization, or post-translational modification of JUB1 via regulatory pathways, altering its function without direct binding. Moreover, JUB1 binds to and regulates the expression of *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A* (*DREB2A*), a key TF involved in the regulation of water deficit–inducible genes, further reinforcing its role in the drought stress response. The second identified TF, AT2G40350, belongs to the same TF family and encodes DREB2H, suggesting a potential functional overlap in regulating plant adaptation to drought conditions. Both TFs play essential roles in activating stress-responsive pathways, highlighting their significance in improving drought tolerance through SL-related signalling. Among the presented data, we also identified DREB2C (AT2G40340), emphasizing the potential role of DREB2 as a key subgroup of TFs regulating water management of SL-related mutants under drought.

These findings underscore the complex network of TFs involved in coordinating drought stress responses and SL signalling. The differential regulation of these TFs in *hvd53a.f* and *hvd14.d* suggests that SL influences drought tolerance through multiple pathways, potentially by modulating ABA

responsiveness, ROS homeostasis, and water-management mechanisms. Further functional characterization of these TFs, particularly their direct interactions with SL-responsive genes, will provide deeper insight into the molecular basis of SL-mediated drought adaptation. Understanding these regulatory networks could pave the way for targeted genetic modifications to improve crop resilience under water-limiting conditions.

Conclusions

Our findings revealed that mutations in the barley SL repressor gene significantly affect plant architecture, flowering time, and photosynthetic performance under non-stressed conditions. The contrasting drought responses of *hvd53a.f* and *hvd14.d* emphasize the complex role of SL signalling in drought adaptation. Although *hvd53a.f* plants exhibited reduced chlorophyll content and lower photosynthetic efficiency under control conditions, they demonstrated enhanced drought tolerance, likely due to alterations in ABA signalling and antioxidant defence mechanisms. We conclude that *hvd53a.f* plants exhibit greater drought tolerance at the cost of lowering photosynthetic efficiency but maintain it at a stably low level. Overall, our study provides new insights into the molecular mechanisms linking SL signalling, photosynthesis, and drought adaptation, which offers potential targets for crop improvement strategies to enhance stress resilience.

Materials and Methods

Gene identification and expression profile analyses

The protein sequence of rice OsD53 SL repressor (LOC_Os11g01330) was blasted against the 'all proteins Morex v3' dataset using the IPK Galaxy Web server (https://galaxy-web.ipk-gatersleben.de/). The expression profiles of *HvD53A* and *HvD53B* were obtained from BarleyExpDB (http://barleyexp.com/; Li et al. 2023) using gene IDs (HORVU.MOREX.r3.4HG0354980 and HORVU.MOREX.r3.5HG0466140) and the PRJEB14349 repository [RNA-Seq of 16 developmental stages of barley (*Morex cultivar*)].

TILLING strategy

TILLING screening was performed on 7680 M2 plants from the *HorTILLUS* population using a pair of primers for each gene (HvD53A_F: TCCTGTCATCCTGGCCTAAC, HvD54A_R: GCCTCTCCATTGACTTGCAC; *HvD53B*_F: TACAGCTGACCAGGGAGGTC, *HvD53B*_R: GAGGCTCAGGTCCAGATGAA) according to a previously described protocol (Szurman-Zubrzycka et al. 2018). Briefly, PCR was performed on eight-fold DNA pools, followed by the formation of heteroduplexes. Next, 2 μl of PCR products was mixed with 2 μl of Celery Juice Extract containing Cel-I enzyme (Till et al. 2006). The mixture was incubated at 45°C for 30 min and then diluted by adding 20 μl of 0.1 × TE. Capillary electrophoresis of the diluted products was run on a Fragment Analyzer 5300 (Agilent). All potential mutations were confirmed using Sanger sequencing.

Plant material, growth conditions, and drought stress

Mutant *hvd14.d*, disturbed in SL signalling, was isolated from the *HorTILLUS* population, as described above (Marzec et al. 2016). The *hvd14.d* mutant carried a substitution (G725A) in the HvDWARF14 gene (NCBI accession number: KP069479) that encodes the SL receptor. Phenotypic analyses were performed on plants grown in the greenhouse under controlled conditions (20/18°C day/night, 16/8 h photoperiod, and 420 $\mu E\, m^{-2}\, s^{-1}$ light intensity). A single surface sterilized with a 20% bleach solution and grains was placed in a pot (15 × 15 × 13 cm) filled with a mixture of vermiculite and soil (1,1).

Drought was induced according to a previously described protocol (Daszkowska-Golec et al. 2019). Briefly, 15 grains were sown in boxes (400 mm × 140 mm × 175 mm) filled with soil containing a sandy loam and sand mixture. Eight boxes were prepared. Plants were grown in a greenhouse (20/18°C day/night, 16/8 h photoperiod, and 420 $\mu E\, m^{-2}\, s^{-1}$ light intensity) for 10 days after sowing (DAS) under optimal water conditions (14% vwc). Next, soil moisture was decreased in four boxes per genotype by withholding irrigation. At 15 DAS, when the soil moisture decreased to 3%, the plants were moved into a growth chamber (25°C/20°C day/night, with a 16/8 h photoperiod and 420 $\mu Em^{-2}\, s^{-1}$ light intensity), and severe drought stress (3%–1.5% vwc) was applied for 10 days (16–25 DAS). Control plants were grown under the same conditions with an optimal water supply (14% vwc), parallel to the drought-treated plants. The soil moisture was measured daily using a time-domain reflectometer (TDR) EasyTest (Institute of Agrophysics, Polish Academy of Sciences).

Plant phenotyping and parameter measurement

Shoot branches were counted weekly for each plant. Plant height was measured only in mature plants. The chlorophyll content was determined using a Dualex Scientific+ chlorophyll metre (Force-A, France). The fluorescence of chlorophyll a was measured using a plant efficiency analyser (PocketPEA fluorimeter, Hansatech Instruments Ltd, England) and used to calculate the parameters of photosynthetic efficiency (Kalaji et al. 2011). RWC was calculated according to the formula: RWC (%) = (fresh weight—dry weight)/(turgid—dry weight) × 100 (Barrs and Weatherley 1962).

Transcriptome analysis

RNA was isolated from four biological replicates; each replicate contained 2 cm-long sections of the second leaf, positioned 3 cm below the leaf tip, collected from three independent plants using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific, catalogue number: AM1560). Library construction and sequencing (150-nt paired-end reads) on the Illumina NovaSeq™ 6000 platform was performed by the Novogene Genomics Service (Cambridge, UK). The Novogene Genomics Service also provided basic data analysis using the RNAseq pipeline. Genes with an adjusted *P*-value $< .05$ and log2FC ≥ 1 or ≤ -1 were considered differentially expressed.

Ultrastructure analysis

Ultrastructure analysis was performed as described previously (Marzec et al. 2020). Briefly, for histological and ultrastructural analysis 2 mm two sections of the second leaf of five different WT and *hvd53a.f* plants were used for combined conventional and microwave-proceeded fixation, dehydration, and resin embedding in a PELCO BioWave® 34 700-230 (Ted Pella, Inc., Redding, CA, USA). Semi-thin sections with a thickness of ~2.5 μm were mounted on slides and stained for 2 min with 1% methylene blue/1% Azur II in 1% aqueous borax at 60°C before light microscopical examination in a Zeiss Axio Imager M2 microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany). Ultra-thin sections with a thickness of ~70 nm were cut with a diamond knife, transferred onto TEM-grids and contrasted in a LEICA EM STAIN (Leica Microsystems, Vienna, Austria) with uranyl acetate and Reynolds' lead citrate prior to analysis using a Tecnai Sphera G2 transmission electron microscope (FEI, Eindhoven, Netherlands) at 120 kV.

Gene ontology enrichment analysis

ShinyGO 0.81 (https://bioinformatics.sdstate.edu/go/) was used for GO enrichment analysis, with an FDR cutoff set to 0.05 and the pathway dataset set to GO BP. *Hordeum vulgare* TRITEX genes Morex_V2_scaf were used as references.

Promoter sequences analysis and identification of TF

For promoter sequence analysis, the 1500 bp before the codon START ('Flank Gene') of DEGs were downloaded using the BioMart tool (https://plants.ensembl.org/index.html) from the '*H. vulgare* TRITEX gene (Morex_V2_scaf) dataset. Obtained files were used as input to identify potential regulatory interactions between TF and promoter sequences by PlantRegMap 'Regulatory prediction' (https://plantregmap.gao-lab.org/), parallel with sorting out the TF that possess over-represented targets in the input gene set. Arabidopsis homologues of identified barley TF were selected using the Plant Transcription Factor Database (https://planttfdb.gao-lab.org/).

Protein–protein interaction prediction using AlphaFold2

To evaluate potential physical interactions between the SL signalling repressor D53 and selected TFs (CCA1 and JUB1), protein structure predictions were performed using AlphaFold2-based modelling implemented in ColabFold v1.5.2 (https://colabfold.com). Full-length amino acid sequences of the barley proteins were retrieved from the NCBI and Plant Transcription Factor Database (PlantTFDB) (https://planttfdb.gao-lab.org/) and used as input. The 'alphafold2_multimer_v3' mode was selected to model protein–protein complexes, and five ranked models were generated for each pairwise interaction. Model quality was assessed using the inter-chain predicted TM-score (ipTM) and predicted TM-score (pTM) values. Final interpretation was based on ipTM values, where a score above 0.7 typically indicates a confident prediction of direct interaction.

DAB staining against hydrogen peroxide

Leaf fragments from ten individual plants were pooled into a single Falcon tube containing a staining solution prepared according to the manufacturer's instructions (DAB Substrate Kit, Thermo Fisher Scientific), supplemented with 0.05% (v/v) Tween 20. The samples were incubated in the dark on a shaker (80–100 rpm) for eight hours. Following staining, the tissue was subjected to a bleaching step using a solution of ethanol, acetic acid, and glycerol (3:1:1, v/v/v) for 20 min at 95°C. Subsequently, samples were rinsed in fresh bleaching solution for an additional 30 min and then photographed.

Supplementary Data

Supplementary Data is available at *PCP* online.

Author Contributions

M.Ma.: Conceptualization; M.K., W.B., A.D.G., B.C., I.F., M.Me., G.H., J.K., P.B.B., M.Ma.: Investigation; M.K., M.Ma.: Writing—original draft; M.K., W.B., A.D.G., B.C., I.F., M.Me., G.H., J.K., P.B.B., M.Ma.: Writing—review & editing; M.Ma., M.Me.: Funding acquisition. All authors read and approved the manuscript.

Conflict of Interest

The authors declare no competing interests.

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Data Availability

Transcriptomic data repository ID: E-MTAB-14857.

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Regular Paper

Rozdział IV

Streszczenie w języku polskim

Strigolaktony (SL) to roślinne hormony o szerokim spektrum działania, odgrywające kluczową rolę w regulacji architektury roślin oraz w odpowiedzi na stresy środowiskowe. Pomimo coraz lepszego poznania szlaku ich biosyntezy i percepcji, molekularne mechanizmy działania SL pozostają nadal niejasne. Celem rozprawy doktorskiej było pogłębienie wiedzy na temat roli SL w regulacji wzrostu i rozwoju jęczmienia zwyczajnego (*Hordeum vulgare*), ze szczególnym uwzględnieniem interakcji SL z innymi hormonami oraz ich funkcji w odpowiedzi na stres suszy. W tym celu przeprowadzono badania zmierzające do identyfikacji czynników transkrypcyjnych zależnych od strigolaktonów.

W pracy wykorzystano dwa mutanty jęczmienia: *hvd14.d*, z mutacją w genie receptora SL (*DWARF14*), oraz zidentyfikowany w ramach rozprawy mutant *hvd53a.f*, z mutacją w genie represora SL (*DWARF53*). Przeprowadzono kompleksowe fenotypowanie tych linii w warunkach kontrolnych oraz stresu suszy, analizując m.in. rozkrzewienie, rozwój systemu korzeniowego, dynamikę wzrostu, zawartość chlorofilu, aktywność fotosyntetyczną oraz poziom reaktywnych form tlenu (ROS). Ujawniono wyraźne różnice pomiędzy mutantami – *hvd14.d* wykazywał zmniejszoną tolerancję na suszę i silnie rozkrzewiony pęd, natomiast *hvd53a.f* prezentował kontrastujący fenotyp związany z ograniczoną liczbą źdźbeł oraz zwiększoną adaptacją do stresu suszy.

W celu identyfikacji mechanizmów molekularnych odpowiedzialnych za obserwowane różnice, przeprowadzono analizy transkryptomiczne i proteomiczne, uwzględniając różne stadia rozwojowe i warunki wzrostu jęczmienia. Uzyskane wyniki ujawniły liczne geny o zróżnicowanej ekspresji (DEG) oraz białka o zmiennym poziomie akumulacji (DAP), związane z sygnalizacją hormonalną, metabolizmem redoks i gospodarką wodną. Wykazano, że *hvd53a.f* aktywuje szlaki związane z metabolizmem oksylipin, glutationu oraz białek z rodziny DEHYDRIN, co może stanowić podstawę zwiększonej tolerancji tej linii na stres suszy.

Ponadto, wykorzystując metody *in silico*, przeprowadzono analizę motywów *cis*-regulatorowych w promotorach genów zależnych od SL oraz ich homologów w *Arabidopsis thaliana*, co pozwoliło na zaproponowanie potencjalnych czynników

transkrypcyjnych, które do tej pory nie były łączone ze SL. W tym czynniki transkrypcyjne kluczowe dla regulacji cyklu okołodobowego.

W warunkach stresu suszy zauważono wyraźnie odmienne strategie adaptacyjne pomiędzy analizowanymi genotypami. Mutant *hvd14.d*, pozbawiony funkcjonalnego receptora SL, wykazywał nasilone objawy stresu wodnego, w tym silniejszą akumulację ROS, obniżoną zawartość chlorofilu oraz spadek wydajności procesu fotosyntezy. Z kolei rośliny *hvd53a.f* utrzymywały stabilną aktywność fotosyntetyczną, efektywniejszą gospodarkę wodną oraz zwiększoną aktywność antyoksydacyjną, co sugeruje większy potencjał adaptacyjny tego mutanta.

Uzyskane wyniki dostarczają nowych dowodów na udział SL w koordynacji wzrostu, rozwoju i odpowiedzi na stres suszy u jęczmienia. Praca stanowi istotny wkład w zrozumienie funkcji SL u roślin uprawnych oraz otwiera nowe perspektywy dla wykorzystania tej wiedzy w hodowli odmian lepiej przystosowanych do zmian klimatycznych.

Rozdział V

Streszczenie w języku angielskim

Strigolactones (SL) are plant hormones with a broad spectrum of activity, playing a key role in the regulation of plant architecture and responses to environmental stresses. Despite increasing knowledge of their biosynthesis and perception pathways, the molecular mechanisms of SL action remain unclear. The aim of this doctoral dissertation was to deepen our understanding of the role of SL in the regulation of growth and development of barley (*Hordeum vulgare*), with particular emphasis on SL interactions with other hormones and their function in drought stress responses. To this end, studies were conducted to identify SL-dependent transcription factors.

Two barley mutants were used in this work: *hvd14.d*, carrying a mutation in the SL receptor gene (*DWARF14*), and *hvd53a.f*, identified within the scope of this dissertation, carrying a mutation in the SL repressor gene (*DWARF53*). Comprehensive phenotyping of these lines was performed under control and drought stress conditions, analyzing, among other traits, tillering, root system development, growth dynamics, chlorophyll content, photosynthetic activity, and reactive oxygen species (ROS) levels. Clear differences were observed between the mutants – *hvd14.d* exhibited reduced drought

tolerance and highly branched shoots, whereas *hvd53a.f* displayed a contrasting phenotype characterized by a limited number of tillers and increased adaptation to drought stress.

To identify the molecular mechanisms responsible for the observed differences, transcriptomic and proteomic analyses were conducted, considering different developmental stages and growth conditions of barley. The results revealed numerous differentially expressed genes (DEG) and differentially accumulated proteins (DAP) associated with hormonal signaling, redox metabolism, and water management. It was shown that *hvd53a.f* activates pathways related to oxylipin and glutathione metabolism as well as DEHYDRIN family proteins, which may underlie the increased drought tolerance of this line.

Furthermore, using *in silico* approaches, an analysis of *cis*-regulatory motifs in the promoters of SL-dependent genes and their homologs in *Arabidopsis thaliana* was performed, which allowed the proposal of potential transcription factors not previously associated with SLs. These included transcription factors crucial for the regulation of the circadian cycle.

Under drought stress conditions, clearly different adaptive strategies were observed between the analyzed genotypes. The *hvd14.d* mutant, lacking a functional SL receptor, exhibited intensified drought stress symptoms, including stronger ROS accumulation, reduced chlorophyll content, and decreased photosynthetic performance. In contrast, *hvd53a.f* plants maintained stable photosynthetic activity, more efficient water management, and increased antioxidant activity, suggesting a higher adaptive potential of this mutant.

The obtained results provide new evidence for the involvement of SLs in coordinating growth, development, and drought stress responses in barley. This work represents a significant contribution to understanding SL function in crop plants and opens new perspectives for applying this knowledge in breeding varieties better adapted to climate change.

ROZDZIAŁ VI

Oświadczenia doktoranta i współautorów

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

05.09.2025, Katowice

mgr Magdalena Korek

Instytut Biologii, Biotechnologii i Ochrony Środowiska

Wydział Nauk Przyrodniczych

Uniwersytet Śląski

Oświadczenie doktoranta dotyczące udziału w postępowaniu prac stanowiących rozprawę doktorską

We wszystkich pracach wchodzących w skład rozprawy doktorskiej jestem pierwszą autorką, a w większości z nich pełnię również funkcję autora korespondującego, co wskazuje na mój wiodący udział w ich powstaniu. Poniżej znajduje się szczegółowy opis mojego wkładu w przygotowanie każdego z artykułów.

1. **Korek M.** and Marzec M. 2023. Strigolactones and abscisic acid interactions affect plant development and response to abiotic stresses. BMC Plant Biology 23: 314

- Przeprowadziłam systematyczny przegląd i selekcję istotnych artykułów naukowych, stanowiących podstawę merytoryczną niniejszej pracy przeglądowej.
- Opracowałam i napisałam sekcje opisujące szlaki biosyntezy i sygnalizacji strigolaktonów oraz kwasu abscysynowego, jak również ich wzajemne interakcje w warunkach stresu abiotycznego. Moim zadaniem było także zintegrowanie fragmentów tekstu przygotowanych przez współautora w spójną całość.
- Byłam zaangażowana we wprowadzanie wszystkich niezbędnych poprawek i zmian merytorycznych, przygotowując wersję poprawioną w odpowiedzi na uwagi recenzentów.
- Zaprojektowałam i przygotowałam większość schematów, tabel i rysunków znajdujących się w manuskrypcie, które w przejrzysty sposób ilustrują omawiane zagadnienia.

2. **Korek M.** and Marzec M. 2024. Chapter 4 - An update on strigolactone signaling in plants. In Strigolactones – Synthesis, Application and Role in Plants Edited by Bashri, G., Hayat, S., and Bajguz, A. pp. 53–73 Academic Press.

- Przeprowadziłam systematyczny przegląd i selekcję istotnych artykułów naukowych, stanowiących podstawę merytoryczną niniejszego rozdziału.
- Opracowałam i napisałam sekcje dotyczące sygnalizacji fitohormonów, transdukcji sygnału strigolaktonów oraz czynników transkrypcyjnych zależnych od tego hormonu. Moim zadaniem było także zintegrowanie fragmentów tekstu przygotowanych przez współautora w spójną całość.
- Byłam zaangażowana we wprowadzanie wszystkich niezbędnych poprawek i zmian merytorycznych, przygotowując wersję poprawioną w odpowiedzi na uwagi recenzentów.
- Zaprojektowałam i przygotowałam większość schematów, tabel i rysunków znajdujących się w manuskrypcie, które w przejrzysty sposób ilustrują omawiane zagadnienia.

3. **Korek M.**, Uhrig RG., Marzec M. 2024. Strigolactone insensitivity affects differential shoot and root transcriptome in barley. Journal of Applied Genetics 66: 15-28

- Brałam aktywny udział w opracowaniu koncepcji badań oraz szczegółowego planu eksperymentalnego.
- Samodzielne prowadziłam hodowlę roślin oraz realizowałam zaplanowane eksperymenty.
- Analizowałam dane pochodzące z obserwacji fenotypowych oraz analizy transktyptomu.
- Byłam odpowiedzialna za integrację wszystkich uzyskanych wyników.
- Napisałam części manuskryptu dotyczącą dyskusji wyników oraz byłam zaangażowana w krytyczną rewizję i uzupełnianie tekstu na wszystkich etapach powstawania pracy
- Zaprojektowałam i przygotowałam większość schematów, tabel i rysunków znajdujących się w manuskrypcie, które w przejrzysty sposób ilustrują omawiane zagadnienia.
- Pozyskałam część środków przeznaczonych na uiszczenie opłaty publikacyjnej w wersji Open Access. Środki pozyskane były w ramach konkursu ogłoszonego przez Szkołę Doktorską Uniwersytetu Śląskiego: National Agency for Academic Exchange under the STER program – Internationalization of Doctoral Schools, project: International from the beginning – wsparcie umiędzynarodowienia (BPI/STE/2023/1/00012/U/00001)

Korek M., Mehta D., Uhrig GR., Daszkowska-Golec A., Novak O., Buchcik W., Marzec M. 2025. Strigolactone insensitivity affects the hormonal homeostasis in barley. Scientific Reports 15: 9375

- Brałam aktywny udział w opracowaniu koncepcji badań oraz szczegółowego planu eksperymentalnego.
- Samodzielne prowadziłam hodowlę roślin oraz realizowałam zaplanowane eksperymenty.
- Analizowałam dane pochodzące z obserwacji fenotypowych oraz analizy transktyptomu.
- Byłam odpowiedzialna za integrację wszystkich uzyskanych wyników.
- Napisałam części manuskryptu obejmujące wstęp tematyczny, opis wyników dotyczących zmian w transkryptomie i proteomie, selekcję czynników transkrypcyjnych potencjalnie zależnych od SL oraz dyskusję otrzymanych rezultatów.
- Byłam zaangażowana w krytyczną rewizję i uzupełnianie tekstu na wszystkich etapach powstawania pracy
- Zaprojektowałam i przygotowałam większość schematów, tabel i rysunków znajdujących się w manuskrypcie, które w przejrzysty sposób ilustrują omawiane zagadnienia.

5. **Korek M.**, Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

- Brałam aktywny udział w opracowaniu koncepcji badań oraz szczegółowego planu eksperymentalnego.
- Samodzielne prowadziłam hodowlę roślin oraz realizowałam zaplanowane eksperymenty.
- Analizowałam dane pochodzące z obserwacji fenotypowych oraz analizy transktyptomu.
- Byłam odpowiedzialna za integrację wszystkich uzyskanych wyników.
- Napisałam części manuskryptu dotyczące (wymienić) oraz byłam zaangażowana w krytyczną rewizję i uzupełnianie tekstu na wszystkich etapach powstawania pracy
- Zaprojektowałam i przygotowałam większość schematów, tabel i rysunków znajdujących się w manuskrypcie, które w przejrzysty sposób ilustrują omawiane zagadnienia.

Korek

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

Katowice, 05.09.2025

dr hab. Marek Marzec
Instytut Biologii, Biotechnologii i Ochrony Środowiska
Wydział Nauk Przyrodniczych
Uniwersytet Śląski

Oświadczenie współautora dotyczące udziału w postępowaniu prac stanowiących rozprawę doktorską

Oświadczam, że mój udział w przygotowaniu publikacji wchodzących w skład rozprawy doktorskiej mgr Magdaleny Korek, polegał przede wszystkim na opracowywaniu koncepcji badań, planowaniu doświadczeń, udziale w przeprowadzeniu wybranych eksperymentów, dyskusji otrzymanych wyników, przygotowaniu niektórych fragmentów opublikowanych tekstów oraz krytycznej rewizji manuskryptów. Ponadto byłem odpowiedzialny za pozyskanie środków finansowych, które umożliwiły prowadzenie badań oraz ich opublikowanie. Wkład ten dotyczy wszystkich poniżej wymienionych prac:

1. **Korek M.** and Marzec M. 2023. Strigolactones and abscisic acid interactions affect plant development and response to abiotic stresses. BMC Plant Biology 23: 314

2. **Korek M**. and Marzec M. 2024. Chapter 4 - An update on strigolactone signaling in plants. In Strigolactones. Edited by Bashri, G., Hayat, S., and Bajguz, A. pp. 53–73 Academic Press.

3. **Korek M**., Uhrig RG., Marzec M. 2024. Strigolactone insensitivity affects differential shoot and root transcriptome in barley. Journal of Applied Genetics 66: 15-28

4. **Korek M**., Mehta D., Uhrig GR., Daszkowska-Golec A., Novak O., Buchcik W., Marzec M. 2025. Strigolactone insensitivity affects the hormonal homeostasis in barley. Scientific Reports 15: 9375

5. **Korek M**., Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

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

Katowice, 5 września 2025

dr hab. Agata Daszkowska-Golec

Instytut Biologii, Biotechnologii i Ochrony Środowiska

Wydział Nauk Przyrodniczych

Uniwersytet Śląski

Oświadczenie współautora dotyczące udziału w postępowaniu prac stanowiących rozprawę doktorską

1. **Korek M**., Mehta D., Uhrig G.R., Daszkowska-Golec A., Novak O., Buchcik W., Marzec M. 2025. Strigolactone insensitivity affects the hormonal homeostasis in barley. Scientific Reports 15, 9375

Mój udział w powstaniu publikacji polegał na bioinformatycznej obróbce surowych odczytów uzyskanych po sekwencjonowaniu RNA wyizolowanego z materiału roślinnego. Procedura obejmowała ocenę jakości odczytów, trymowanie sekwencji adapterów i odfiltrowanie niskiej jakości odczytów oraz mapowanie i zliczenie odczytów w odniesieniu do referencyjnego transkryptomu jęczmienia. Kolejno przeprowadziłam analizę różnicowej ekspresji genów. Dodatkowo zaangażowana byłam w korekcję przygotowanego manuskryptu.

2. **Korek M**., Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

Mój udział w powstaniu publikacji obejmował przeprowadzenie analizy wpływu mutacji w genie *HvD53A* oraz *HvD14* na wydajność fotosyntezy. Zawartość chlorofilu, liczba centrów reakcji, indeks wydajności fotosyntezy oraz wskaźnik rozproszenia energii zmierzone i obliczone zostały u roślin rosnących w warunkach kontrolnych oraz poddanych stresowi suszy. Opracowana została przez mnie także analiza statystyczna otrzymanych wyników. Ponadto, mój wkład w powstanie publikacji obejmował korektę edytorską przygotowanego manuskryptu.

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

Düsseldorf, September 5, 2025

Dr. Goetz Hensel

Heinrich Heine University Düsseldorf,

Faculty of Mathematics and Natural Sciences,

Centre for Plant Genome Engineering,

Universitätsstr. 1

40225 Düsseldorf

Germany

Author's statement about participation in manuscript preparation

1. **Korek M**., Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

My contribution involved the conceptualization of the research and the critical revision of the manuscript text.

Dr. Goetz Hensel

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

Gatersleben, 5th September 2025

Jochen Kumlehn, Dr.

Leibniz Institute of Plant Genetics and Crop Plant Research (IPK),

Germany

Author's statement about participation in manuscript preparation

1. **Korek M**., Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

My contribution involved the conceptualization of the research and the critical revision of the manuscript text.

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

Gatersleben, 05.09.2025

Dr. Michael Melzer
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Corrensstrasse 3
06466 Seeland, OT-Gatersleben
Germany

Author's statement about participation in manuscript preparation

1. **Korek M**., Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

I, Michael Melzer, hereby confirm that my work in connection with the above-mentioned publication dealt with the histological and ultrastructural analysis of hvd53a.f mutants and the barley cultivar Sebastian.

Michael Melzer

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

Olomouc, September 5 2025

Ondřej Novák, Professor

Faculty of Science, Palacký University

and Institute of Experimental Botany, The Czech Academy of Sciences

Olomouc, Czech Republic

Author's statement about participation in manuscript preparation

My involvement in the creation of the publication was connected with laboratory part of phytohormone profiling by targeted metabolomics.

1. **Korek M.,** Mehta D., Uhrig G.R., Daszkowska-Golec A., Novak O., Buchcik W., Marzec M. 2025. Strigolactone insensitivity affects the hormonal homeostasis in barley. Scientific Reports 15, 9375

Ondřej Novák

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

Edmonton, Alberta, Canada
2025-09-05

Department of Biological Sciences
University of Alberta
Edmonton, Alberta, Canada
Canada

Author's statement about participation in manuscript preparation

Korek M., Uhrig R.G. & Marzec M. 2025. Strigolactone insensitivity affects differential shoot and root transcriptome in barley. Journal of Applied Genetics 66, 15–28

My contribution involved proteome processing, proteomic data analysis, and manuscript revision.

Korek M., Mehta D., Uhrig G.R., Daszkowska-Golec A., Novak O., Buchcik W., Marzec M. 2025. Strigolactone insensitivity affects the hormonal homeostasis in barley. Scientific Reports 15, 9375

My contribution involved manuscript revision.

Prof. Dr. R. Glen Uhrig

Associate Professor
Department of Biological Sciences
University of Alberta

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

Katowice, 10.09.2025

mgr Weronika Buchcik,

Instytut Biologii, Biotechnologii i Ochrony Środowiska

Wydział Nauk Przyrodniczych

Uniwersytet Śląski

Oświadczenie współautora dotyczące udziału w postępowaniu prac stanowiących rozprawę doktorską

1. **Korek M.,** Mehta D., Uhrig G.R., Daszkowska-Golec A., Novak O., Buchcik W., Marzec M. 2025. Strigolactone insensitivity affects the hormonal homeostasis in barley. Scientific Reports 15, 9375

Mój udział w powstaniu publikacji polegał na dbaniu o materiał roślinny i jego warunki wzrostu, pomoc przy zbiorze tkanki oraz izolacji materiału genetycznego.

2. **Korek M.**, Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

Mój udział w powstaniu publikacji obejmował opiekę nad materiałem roślinnym i jego warunkami wzrostu, pomoc przy zbiorze tkanki oraz izolacji materiału genetycznego, a także wsparcie w analizach bioinformatycznych.

Weronika Buchcik

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

Katowice, 05.09.2025

dr Beata Chmielewska,
Instytut Biologii, Biotechnologii i Ochrony Środowiska
Wydział Nauk Przyrodniczych
Uniwersytet Śląski

Oświadczenie współautora dotyczące udziału w postępowaniu prac stanowiących rozprawę doktorską

Mój udział w powstanie publikacji był związany z przeprowadzeniem analizy TILLING, mającej na celu identyfikację mutacji w genach *HvD53A* i *HvD53B*.

1. **Korek M.**, Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

Beata Chmielewska

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

Gatersleben, 09/09/2025

Irene M. Fontana, MSc,

Leibniz Institute of Plant Genetics and Crop Plant Research (IPK),

Germany

Author's statement about participation in manuscript preparation

1. **Korek M.**, Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

My involvement in the creation of the publication was connected with histological and ultrastructural analysis of *hvd53a.f* mutant and Sebastian plants.

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

Philip B Brewer, Professor

La Trobe Institute for Sustainable Agriculture and Food,

Australian Research Council Centre of Excellence in Plants for Space,

La Trobe University,

Australia

Author's statement about participation in manuscript preparation

1. **Korek M**., Buchcik W., Chmielewska B., Daszkowska-Golec A., Fontana IM., Melzer M. Hensel G., Kumlehn J., Brewer PB., Uhrig GR., Marzec M. 2025. The cost of survival: mutation in a barley strigolactone repressor HvD53A impairs photosynthesis but increases drought tolerance. Plant and Cell Physiology. DOI: 10.1093/pcp/pcaf095

My contribution involved the conceptualization of the research and the critical revision of the manuscript text.

Nie otrzymano podpisanego oświadczenia od współautora

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

Devang Mehta, Assistant Professor

Department of Biosystems,

KU Leuven (BOFZAP Research Professorship)

Author's statement about participation in manuscript preparation

In the manuscript:

Korek M., Mehta D., Uhrig G.R., Daszkowska-Golec A., Novak O., Buchcik W., Marzec M. 2025. Strigolactone insensitivity affects the hormonal homeostasis in barley. Scientific Reports 15, 9375

my contribution involved proteome processing, proteomic data analysis, and manuscript revision.

Nie otrzymano podpisanego oświadczenia od współautora

ROZDZIAŁ VII

Wykaz aktywności i osiągnięć naukowych

Udział w konferencjach krajowych i międzynarodowych

1. Experimental plant biology at various scales: from molecules to the environment – 20.09.21 – 23.09.21 – KATOWICE. Polish Society of Experimental Plant Botany. **Prezentacja posterowa:** „Crosstalk between SLs and ABA signaling pathway". Magdalena Korek, Agata Daszkowska-Golec, Anna Collin & Marek Marzec

2. Second International E-Conference on Plant Science and Biology - 18.04.2022 - 19.04.2022 – LONDYN. United Research Forum. **Prezentacja posterowa:** "Molecular basis of ABA-decreased sensitivity in barley strigolactone receptor mutant". Magdalena Korek, Agata Daszkowska-Golec & Marek Marzec

3. 7th Edition Of Global Conference On Plant Science And Molecular Biology – 01.09.22 – 02.09.22 – PARYŻ. Magnus Group Conferences. **Prezentacja posterowa:** "Identification of SL-responsive TF in barley". Magdalena Korek, Agata Daszkowska-Golec & Marek Marzec

4. 3rd International Conference on Plant Science and Molecular Biology – 17.05.23 – 19.05.23 – LIZBONA. Massive Group Conferences. **Prezentacja posterowa**: "Analysis of SL-responsive genes in Arabidopsis thaliana". Magdalena Korek, Agata Daszkowska-Golec & Marek Marzec

5. V Dni Młodego Naukowca – 24.10.24 – 25.10.24 – RADZIKÓW. Institute of Plant Breeding and Acclimatization – National Research Institute in Radzików. **Wystąpienie ustne:** "Strigolactone insensitivity affects the hormonal homeostasis in barley". Magdalena Korek, Devang Mehta, Glen R. Uhrig, Agata Daszkowska-Golec, Ondrej Novak, Weronika Buchcik, Marek Marzec

6. Sympozjum Biologia Eksperymentalna Roślin w Polsce – osiągnięcia i wyzwania - 25.10.2024 – WARSZAWA. Polskie Towarzystwo Biologii Eksperymentalnej Roślin. **Prezentacja posterowa**: "Mutation in the SL signaling gene *D14* affects barley branching by impairing the hormonal network". Magdalena Korek, Devang Mehta, Glen R. Uhrig, Agata Daszkowska-Golec, Ondrej Novak, Weronika Buchcik, Marek Marzec

7. 9th edition of the Global Conference on Plant Science and Molecular Biology – 16.09.24 – 18.09.24 – RZYM. Magnus Group Conferences. **Wystąpienie ustne**: "Barley

strigolactone-signalling mutant *hvd14.d* presents a highly-tillered phenotype due to hormonal network alternations". Magdalena Korek, Devang Mehta, Glen R. Uhrig, Agata Daszkowska-Golec, Ondrej Novak, Weronika Buchcik, Marek Marzec

8. Plant Biology Europe 2025, Budapest – 25.06.25 – 28.06.25 – BUDAPESZT. Scientific Committee, the Federation of the European Societies of Plant Biology (FESPB). **Wystąpienie ustne**: "Mutation in a barley strigolactone repressor *HvD53A* impairs photosynthesis but increases drought tolerance". Magdalena KorekWeronika Buchcik, Beata Chmielewska, Agata Daszkowska-Golec, Irene M. Fontana, Michael Melzer, Goetz Hensel, Jochen Kumlehn, Philip B. Brewer, Glen R. Uhrig, Marek Marzec.

9. 12th Conference of the Polish Society of Experimental Plant Biology – 09.09.25 – 12.09.2025 – WARSZAWA. Polskie Towarzystwo Biologii Eksperymentalnej Roślin (PTBER). **Wystąpienie ustne**: "The cost of survival: Barley *HvD53A* mutation boosts drought tolerance at the expense of Photosynthesis". Magdalena KorekWeronika Buchcik, Beata Chmielewska, Agata Daszkowska-Golec, Irene M. Fontana, Michael Melzer, Goetz Hensel, Jochen Kumlehn, Philip B. Brewer, Glen R. Uhrig, Marek Marzec.

Dodatkowe aktywności popularyzatorskie, warsztaty, staże

1. Stypendystka projektu OPUS-19 finansowanego przez Narodowe Centrum Nauki: „Investigation of molecular mechanism underlying cross talk between strigolactones and abscisic acid signaling pathways" pod kierownictwem dr hab. Marka Marca. Okres realizacji: 01.03.2021 – 31.12.2024

2. Uczestniczka "13th Poznań Summer School of Bioinformatics" – 05.07.2021 – 09.07.2021

3. Uczestniczka kursu "RNA-seq Results Explained: what you can expect from the analysis" – 17.02.2022

4. Uczestniczka kursu "'A beginner's guide to DNA sequencing and its applications" – 17.03.2022

5. Staż zagraniczy w instytucie naukowym The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Niemcy, Poznanie technik izolacji i kultur protoplastów jęczmienia. 7.11.2022 – 20.11.2022

6. Uczestniczka warsztatu „Becoming a researcher” w wydarzeniu Transform4Europe Mobility Week na Uniwersytecie w Sofii - 25.09.2023 - 1.10.2023

7. Aktywna uczestniczka 6, 7, 8 edycji Śląskiego Festiwalu Nauki – 03.12.22 – 05.12.2022, 09.12.2023 – 11.12.2023, 07.12.2024 – 09.12.2024

8. Aktywna uczestniczka XI, XII, XIII Ogólnopolskiej Nocy Biologów organizowanej przez Wydział Nauk Przyrodniczych UŚ – 21.01.2022, 13.01.2023, 26.01.2024