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Physiologische Untersuchungen zur reproduktiven Kältetoleranz beitragender Mechanismen bei Sorghum (*Sorghum bicolor* L. Moench)

DISSERTATION

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Abkürzungsverzeichnis

ABA – Abscisinsäure

BCNAM – Back-cross Nested Association Mapping

BPH – Best-Parent-Heterosis

CMS – Cytoplasmatische Männliche Sterilität

GA – Gibberellinsäure

GCA – General Combining Ability (allgemeine Kombinationsfähigkeit)

GS – Genomische Selektion

JA – Jasmonsäure

JAIle – Jasmonsäure-Isoleucin

MeJA – Methyljasmonat

NAM – Nested Association Mapping

NGS – Next-Generation Sequencing

oTS – organische Trockensubstanz

PHI – Panicle Harvest Index

QTL – Quantitative Trait Loci

RAD-seq – Restriction site Associated DNA sequencing

SCA – Specific Combining Ability (spezifische Kombinationseignung)

1 Einleitung

Die Bedeutung der Kältetoleranz in Sorghum für die Ausweitung des Anbaus in gemäßigte Breiten zieht zunehmend wissenschaftliches und landwirtschaftliches Interesse auf sich. Mit der stetig steigenden Bevölkerungsdichte nimmt die Verfügbarkeit fruchtbarer Böden bei zunehmender Bodendegradierung ab, was eine ausreichende Produktion von Nahrungs- und Futtermitteln vor neue Herausforderungen stellt (Bindraban et al. 2012). Sorghum, eines der weltweit wichtigsten Getreide für die Produktion von Nahrungs- und Futtermitteln, ist für seine hohe Anpassungsfähigkeit an verschiedene Umweltbedingungen bekannt (Doggett 1988; Berenji und Dahlberg 2004; Zheng et al. 2011). Ursprünglich aus den halbtrockenen Regionen Afrikas stammend, zeichnet sich diese Pflanze durch eine hohe Trockentoleranz, effiziente Wasser- und Nährstoffnutzung, Resistenz gegen Diabrotica virgifera und die Fähigkeit aus, auf marginalen Böden zu gedeihen, was ihr einen Wettbewerbsvorteil im Hinblick auf den Klimawandel verschafft (Oyediran et al. 2004; Patil 2017; Rakshit und Wang 2017). Kältestress stellt jedoch eine erhebliche Herausforderung dar, insbesondere in gemäßigten Regionen, aber auch in tropischen Hochlagen (Pinthus und Rosenblum 1961). Sorghum ist nicht nur im Keimlingsstadium empfindlich, sondern zeigt auch eine erhöhte Anfälligkeit für Kältestress in der reproduktiven Phase. Abhängig von der Schwere des Stresses und der Anfälligkeit des Genotyps kann dies zu einer verminderten oder gar keiner Samenbildung (d. h. vollständigem Ernteverlust) führen (Maulana und Tesso 2013; Chopra et al. 2017; Emendack et al. 2021). Dabei betont die vorhandene Literatur überwiegend die reduzierte Pollenfertilität als den Hauptfaktor für schlechten Kornansatz nach Kältestress (Caddel und Weibel 1971; Downes und Marshall 1971) und somit als Limitierung für den Sorghumanbau in gemäßigten Regionen (Maulana und Tesso 2013). In welchem Ausmaß das weibliche Blütenorgan, genauer gesagt die Rezeptivität des Pistils, durch Kältestress beeinträchtigt wird, ist in diesem Kontext weniger gut untersucht, wurde jedoch bereits diskutiert (Osuna-Ortega, J. et al. 2003). Bei der reproduktiven Kältetoleranz handelt es sich um ein heterotisches und quantitatives Merkmal (Schaffasz et al. 2019b; Chakrabarty et al. 2021). Die an dem Merkmal beteiligten Allele scheinen überwiegend dominant vererbt zu werden. Zusammen mit der hohen phänotypischen Variation und Erblichkeit spricht dies dafür, dass ein nachhaltiger Zuchtfortschritt zu erwarten ist (Schaffasz et al. 2019b).

In Bezug auf die physiologischen, zur Kältetoleranz beitragenden Mechanismen, insbesondere die Regulation von Phytohormonen bei Sorghum, ist das Wissen begrenzt, und es besteht ein

erheblicher Forschungsbedarf. Abscisinsäure (ABA) und Gibberellinsäure (GA) wurden bereits in Reispflanzen als wichtige, an der Kältetoleranz beteiligte Schlüsselhormone identifiziert. Während kältetolerante Reispflanzen durch das Vorhandensein ABA-regulierender genetischer Sequenzen (OSZEP1 und OSNCED3) niedrige ABA-Spiegel aufrechterhalten konnten, war die ABA-Konzentration in kälteempfindlichen Pflanzen signifikant höher (Oliver et al. 2007; Ji et al. 2011; Sharma und Nayyar 2016). Darüber hinaus wird ein erhöhter ABA-Abbau durch Konjugation oder Oxidation bei kältetoleranten Reispflanzen diskutiert, um den ABA-Spiegel zu senken. Hierbei ist die Expression beider ABA-8-Hydroxylase-Gene, ABA8ox1 und ABA8ox2, in kältetoleranten Genotypen höher (Nambara und Marion-Poll 2005; Oliver et al. 2007). Zusätzlich zur ABA-Regulation wurde festgestellt, dass die Aufrechterhaltung eines ausreichend hohen Pools bioaktiver Gibberelline zum Schutz der Pflanze vor Kälteschäden wichtig ist, wobei veränderte Biosynthesewege von GA4 und GA7 diskutiert werden (Sharma und Nayyar 2016). Neben der veränderten ABA- und GA-Regulation wird auch Jasmonsäure eine Schlüsselrolle bei der Beteiligung an Phytohormonen zugeschrieben, die zur Kältetoleranz bei verschiedenen Poaceae-Arten beitragen (Du et al. 2013; Cai et al. 2014). Als Folge erhöhter ABA-Spiegel und unzureichender Pools bioaktiver Gibberellinsäuren bei fehlender Kältetoleranz werden Probleme mit der Hypertrophie der Tapetumzellen und ein abnormales Pollenwachstum beschrieben. Die Unterdrückung des zellwandgebundenen Invertase-Gens und des Monosaccharid-Transporter-Gens stört den Zuckertransport zum Tapetum, was zu einer abnormalen Pollenkornentwicklung und einer geringeren Kornzahl führt (Oliver et al. 2005; Mamun et al. 2006). Im Gegensatz dazu wurde eine reduzierte Fruchtbarkeit des weiblichen Blütenorgans als mögliche Ursache für die verringerte Kornbildung bei kälteempfindlichen Genotypen diskutiert (Osuna-Ortega et al. 2003).

Diese Arbeit zielt darauf ab, ein umfassenderes Verständnis insbesondere der physiologischen Mechanismen im Zusammenhang mit Phytohormonen der reproduktiven Kältetoleranz in Sorghum zu gewinnen, um den Zuchtfortschritt hin zu kältetolerantem Sorghum mit erhöhter Ertragsstabilität in gemäßigten Regionen zu beschleunigen.

2 Grundlagen

2.1 Ökonomische Grundlagen

Mit einem Anbau auf einer Gesamtfläche von 40,7 Millionen Hektar und einer Produktion von 57,6 Millionen Tonnen stellt Sorghum nach Weizen, Reis, Mais und Gerste die fünftwichtigste Getreidekultur weltweit dar. Während in Afrika die Hälfte des Sorghums produziert wird, zeigen sich Amerika und Asien mit 29 % und 14 % als die, nach Afrika, größten Produktionsstätten von Sorghum. Ozeanien und Europa tragen nur 5 % bzw. 1 % zur globalen Sorghumproduktion bei (FAO 2024) (Abb. 1). Der geringflächige Anbau von Sorghum in Europa, welches durch sein gemäßigtes Klima geprägt ist, ist unter anderem auf die Empfindlichkeit von Sorghum gegenüber Kälte zurückzuführen. Die Adaption an kühlere gemäßigte Regionen erscheint jedoch aufgrund der großen Diversität für das Merkmal Kältetoleranz in Zukunft möglich. Die vorhandene Diversität ist der im Gegensatz zu anderen Hirsearten überregionalen Bedeutung von Sorghumhirse geschuldet. Während der Anbau anderer Hirsearten nur regional erfolgte, wird Sorghum seit Jahrtausenden in vielen Ländern sowohl der südlichen als auch der nördlichen Hemisphäre angebaut (SINGH 1985). Seine Vielseitigkeit, welche eine Verwendung für Nahrung, Tierfutter, Weideland, Faser, Brennstoff, Bioethanol, alkoholische Getränke und Baumaterial ermöglicht, macht Sorghum attraktiv für den Anbau in Europa (Hariprasanna und Patil 2015; Kazungu et al. 2023). In den ariden und semiariden Regionen der Welt findet das Getreide jedoch hauptsächlich als Grundnahrungsmittel in Form von Brot, Brei und Kuchen Verwendung (Taylor 2004; Ari Akin et al. 2022). Der überwiegende Anbau in subtropischen und semiariden Gebieten resultiert aus der Fähigkeit von Sorghum, Trockenperioden zu tolerieren, die auf seine hohe Wasser- und Nährstoffnutzungseffizienz zurückzuführen ist. Diese Eigenschaften ermöglichen es, auch unter weniger optimalen Trockenbedingungen zu gedeihen (Zeise und Fritz 2012; Dillon et al. 2007; Patil 2017).

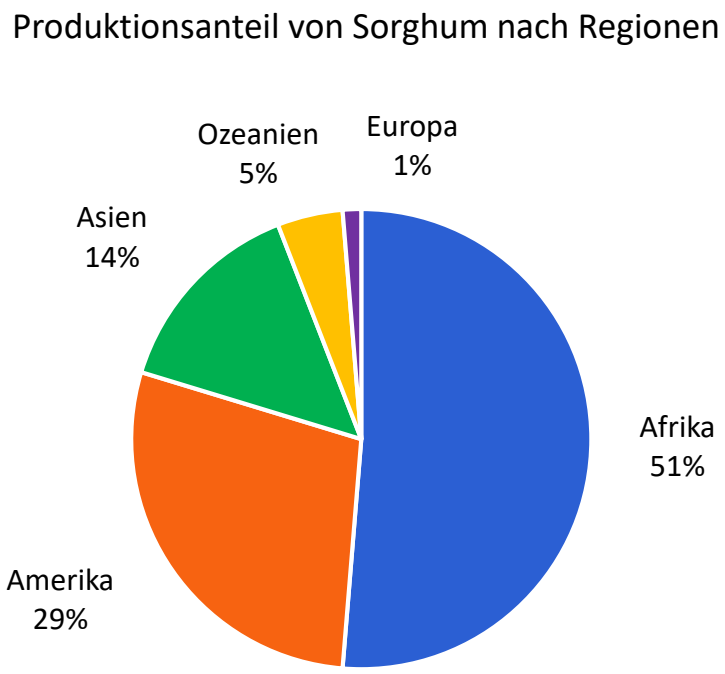

Abbildung 1: Produktionsanteil von Sorghum nach Regionen 2024. Während Afrika mit 51 % führender Sorghum-Produzent ist, folgen Amerika (29 %), Asien (14 %), Ozeanien (5 %) und Europa (1 %) (FAO 2024)

Durch den Anbau an verschiedenen extremen Standorten fällt beim Vergleich von Produktionsmengen und Erträgen der weltweit führenden Länder auf, dass das Ertragsniveau je nach Standort und Kultivierungsmethoden stark variiert. Die fünf größten Produzenten in der Welt sind Nigeria (16,4 %), Sudan (12,6 %), USA (11,5 %), Mexiko (11,4 %) und Äthiopien (10,1 %) (Abb. 2). Zusammen erzeugen sie 62 % der gesamten Weltproduktion. Während Nigeria mit 6,8 Mio. t und Sudan mit 5,3 Mio. t bei Flächenerträgen von nur 1,19 t/ha bzw. 0,75 t/ha Hauptproduzenten sind, generiert die USA als drittgrößter Produzent deutlich höhere Erträge von 3,12 t/ha. Trotz des hohen Ertragspotenzials von 4,8 t/ha ist China mit einer Anbaufläche von 662.000 ha nur siebtgrößter Produzent von Sorghumhirse (FAO 2024).

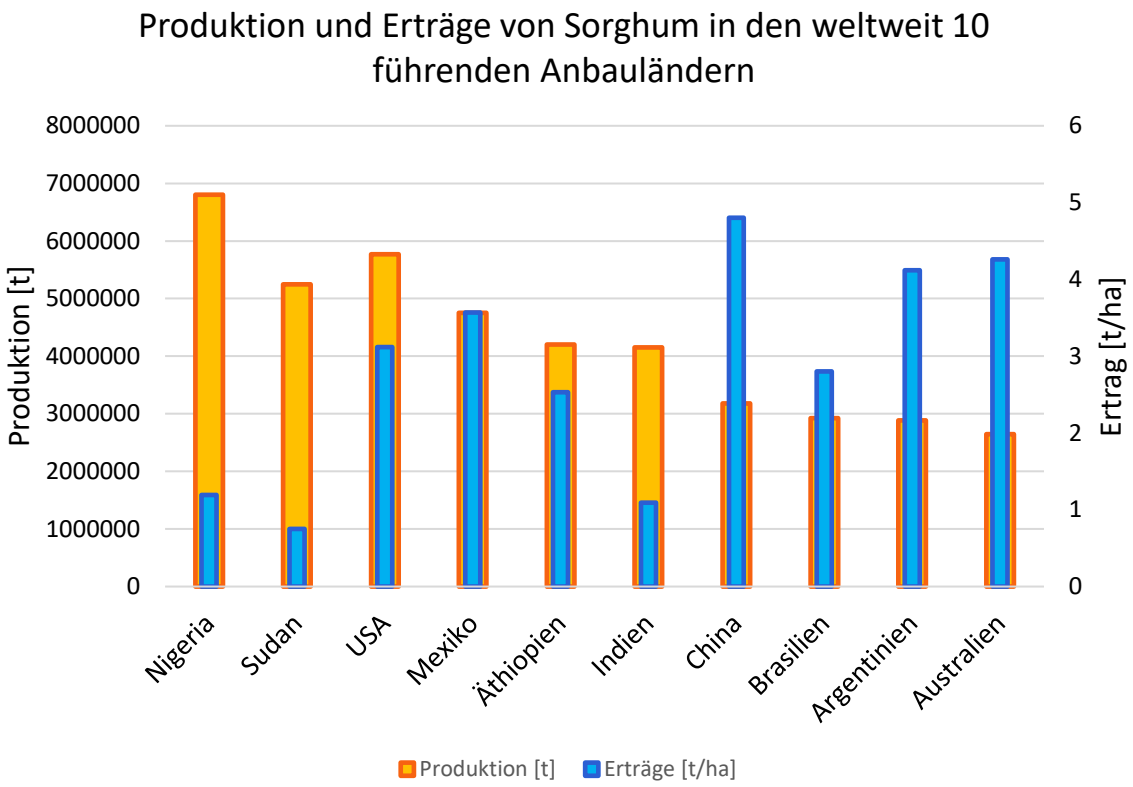

Abbildung 2: Produktion und Erträge von Sorghum der weltweit 10 führenden Anbauländer 2024 (FAO 2024)

2.2 Biologie und Herkunft von Sorghum

Innerhalb der Familie der Poaceae gehört *Sorghum bicolor* [L.] Moench (diploid, 2n = 20) zur Tribus der Andropogoneae und zum Untertribus der Sorghinae. Die Gattung *Sorghum* umfasst insgesamt 25 Arten, die in fünf taxonomische Untergattungen unterteilt sind: Eu-sorghum, Chaetosorghum, Heterosorghum, Parasorghum und Stiposorghum (Dillon et al. 2007). Anhand ihrer Ährchen- und Rispenmorphologie wird *Sorghum* in fünf Grundrassen und zehn intermediäre Rassen unterteilt. Die Grundrassen umfassen *S. bicolor*, *S. guinea*, *S. caudatum*, *S. kafir* und *S. durra* (Madhusudhana et al. 2015). Sobald diese die Reife erreicht haben, lassen sie sich anhand der charakteristischen Merkmale von Spelzen und Rispen unterscheiden.

Sowohl *S. bicolor* als auch *S. guinea* weisen eine offene Rispe auf. Gut unterscheiden lassen sich diese beiden Grundrassen an ihren Spelzen. Während für *S. bicolor* dicke, ledrige Spelzen charakteristisch sind, weist *S. guinea* behaarte, offene und eingerollte Spelzen auf. Die Rispen von *S. caudatum* sind dicht bis leicht geöffnet; die Ährchen sind asymmetrisch, mit flacher oder sogar konkaver Unterseite und gerundeter, gewölbter Oberseite. Die Deckspelzen sind lederartig, behaart und kürzer als das große Korn. Mit einem mittel- bis großwüchsigen Habitus und hoher Kornqualität weist *S. caudatum* herausragende agronomische

Eigenschaften auf, was diese Rasse zu einer der wichtigsten Saatgutquellen für moderne Sorghum-Zuchtprogramme macht. *S. durra* weist eine kompakte Rispe mit ovaler bis länglicher Form auf sowie einen gebogenen Halm unterhalb der Rispe. Die Ährchen sind groß und besitzen leicht pigmentierte Deckspelzen (Hariprasanna und Patil 2015). Durch die Kompaktheit der Rispe ist diese Rasse an Hitze und Trockenheit bestens angepasst (Kimber et al. 2013). Die aufrechten, langgestreckten Rispen von *S. kafir* sind größtenteils semikompakt und zylindrisch, wobei die Äste und die sessilen Ährchen behaart sind. Die Deckspelzen weisen bei Reife einen auffälligen Glanz auf und sind deutlich kürzer als das Korn (Harlan und Wet 1972; Hariprasanna und Patil 2015).

Sorghum vermehrt sich überwiegend durch Selbstbestäubung. In Abhängigkeit von Rispenmorphologie und Wetterbedingungen kann es jedoch bis zu 50 % Fremdbestäubung kommen (Osuna-Ortega et al. 2003).

Abbildung 3: Die fünf Grundrassen von *Sorghum bicolor: bicolor, caudatum, kafir, durra* und *guinea* (eigene Aufnahme, 28.09.2024, Groß Gerau)

Die ältesten datierten Funde lassen darauf schließen, dass die Domestikation von Sorghumhirse bereits 5000–8000 v. Chr. in Nordostafrika oder an der heutigen ägyptisch-

sudanesischen Grenze begann (Rakshit und Wang 2017). Ebenfalls wird vermutet, dass Sorghum bereits um etwa 8000 v. Chr. von Jägern und Sammlern als Nahrungsquelle genutzt wurde (Smith und Frederiksen 2000). Ursprung und Domestikation der Kulturpflanze werden in Afrika verortet, was dementsprechend das größte Diversitätszentrum des wilden als auch des kultivierten *Sorghum bicolor* darstellt (Kimber 2000; Doggett 1988). Neben Afrika gilt der indische Subkontinent als sekundäres Zentrum der genetischen Diversität (Madhusudhana et al. 2015). Die hohe Vielfalt der Hirse lässt sich durch die Verschleppung durch den Menschen, Disruptive Selektion sowie Isolierung und Rekombination in den verschiedenen Lebensräumen Nordafrikas erklären (Doggett 1988). Von dem ursprünglichen Domestikationsgebiet aus kam es im späteren Verlauf zu einer Ausweitung des Anbaus in weitere Regionen Afrikas sowie nach Asien, in den Nahen Osten und bis nach China (Rakshit und Wang 2017).

Ursprünglich ist Sorghum eine photoperiodisch sensitive Kurztagpflanze. Das bedeutet, dass der Zeitpunkt der Blüte stark durch die Länge von Tag und Nacht gesteuert wird und die Pflanze bei kürzer werdenden Tagen in die Blüte geht. In Milo-Sorten wurden vier Loci identifiziert (Ma1, Ma2, Ma3 und Ma4), welche die Blütezeit der Kulturpflanze beeinflussen (Quinby und Karper 1945; Quinby 1966). Rooney und Aydin (1999) konnten zwei weitere Loci identifizieren, die ebenfalls am Blühzeitpunkt beteiligt sind (Ma5, Ma6). Das Reife-Gen Ma1 hat dabei von den bisher bekannten Reife-Genen den größten Einfluss auf den Blühzeitpunkt (Klein et al. 2008). Für die Adaptation an höhere Breiten war eine Mutation am Lokus Ma1 entscheidend, welche eine Blüte unter Langtagbedingungen im Sommer und nicht erst nach der Tag-und-Nacht-Gleiche im Herbst ermöglicht. Dies hat maßgeblich zur frühen Domestikation und Verbreitung von Sorghumhirse in außertropische Regionen beigetragen. Durch die frühere Blüte unter Langtagsbedingungen kann vor dem ersten Frost eine ausreichende Reife erreicht werden (Quinby 1967).

Als eine ursprünglich aus den Tropen und Subtropen stammende C4-Pflanze zählt Sorghumhirse zu den wärmeliebenden Arten. Entsprechend zeigt die Pflanze eine hohe Empfindlichkeit gegenüber niedrigen Temperaturen (Pinthus und Rosenblum 1961). Gleichzeitig ist Sorghum aufgrund seiner Herkunft an trockene Standorte angepasst und zeichnet sich durch eine hohe Effizienz in der Wasser- und Nährstoffnutzung aus (Tari et al. 2013). Sorghum weist bezogen auf die produzierte Trockenmasse eine

Wassernutzungseffizienz von 372 kg cm^{-1} auf. Im Vergleich zu Mais (272 kg cm^{-1}) und Sojabohne (135 kg cm^{-1}) erreicht Sorghum damit die höchsten Werte (Djanaguiraman et al. 2018). Diese Eigenschaften sind sowohl auf den C4-Metabolismus als auch auf das weitläufige, faserige Wurzelsystem zurückzuführen. Ein weiterer Anpassungsmechanismus bei Trockenstress ist die Krümmung der Blätter, wodurch die Transpiration reduziert und Wasserverluste begrenzt werden (Rehm und Espig 1996; Kimber 2000; Becker 2019). Die Blätter und Stängel sind zusätzlich durch eine ausgeprägte Wachsschicht geschützt, welche die Pflanze widerstandfähiger gegen Trockenheit und Insektenbefall macht (Nwanze et al. 1992; Lewandowska et al. 2020).

Welche Merkmale bei der Nutzung von Sorghum präferiert werden, hängt stark von der jeweiligen Anbauregion ab. In gemäßigten Klimazonen werden vor allem photoperiodisch neutrale und eher kleinwüchsige Sorten bevorzugt, da diese eine vereinfachte Ernte ermöglichen. In tropischen Regionen kommen dagegen häufiger photosensitive und hochwüchsige Typen zum Einsatz (Rakshit und Wang 2017). Die Wuchshöhe von Sorghumhirse hat eine Spannweite von Zwergtypen mit 0,5 m bis hin zu 4 Meter hohen Biomasse-Typen. Diese hohe Variation ist auf die vier „Dwarfing-Gene“ DW1, DW2, DW3 und DW4 zurückzuführen, die die Internodienlänge unabhängig voneinander regulieren (Smith 2000; Sleper und Poehlman 2006).

2.3 Nutzung von Sorghum

Sorghumhirse ist durch seine ausgeprägte phänotypische Variabilität eine vielseitig nutzbare Kulturpflanze. Auf Basis der hohen genetischen und morphologischen Diversität haben sich unterschiedliche Nutzungstypen wie Kornsorghum, Zuckersorghum und Futtersorghum entwickelt. Eine eindeutige Abgrenzung ist aufgrund der traditionell bedingten mehrfachen Nutzung eines Pflanzentyps nicht immer möglich (Smith 2000).

In der Subsistenzwirtschaft wird Sorghumhirse häufig als duale oder multifunktionale Kulturpflanze angebaut, während in Industriestaaten die Verwendung spezialisierter, für die maschinelle Landwirtschaft optimierter Zuchtformen forciert wird (Smith 2000). Grundsätzlich dient Sorghum als Quelle für Lebensmittel, Futtermittel, Faserprodukte und energetische Nutzungspfade (Doggett 1988). In den Herkunftsländern ist Sorghumhirse ein

wichtiger Bestandteil der Humanernährung. In den Industrieländern liegt der Fokus auf der Nutzung als Viehfutter (Jambunathan 1980; Gomez et al. 1992).

In der Verwendung als Nahrungsmittel wird das Korn traditionell zu Fladenbrot, gekochtem Brei und anderen Lebensmitteln verarbeitet (Ciacci et al. 2007). Mit ca. 60–75 % Stärke, 8–15 % Protein und 4–6 % Fetten ist die Kornzusammensetzung von Sorghum vergleichbar mit der von Mais und Weizen (Bean et al. 2018). Für an Zöliakie erkrankte Menschen zeigt sich Sorghum als glutenfreies Lebensmittel als eine Alternative, unbedenkliche Nahrungsquelle (Ciacci et al. 2007). Neben der Nutzung als Malz oder Rohfrucht für die Bierherstellung wird Sorghumhirse zu geringen Teilen auch zur Herstellung von Sirup und Süßungsmitteln verwendet (Rooney 1996).

Für die Tierernährung oder in der Nutzung als nachwachsender Rohstoff wird die Hirse als Ganzpflanze genutzt. Hierbei findet die Ernte der Sorghumpflanzen zur Milch- bzw. Teigreife statt. Als Futtermittel findet Sorghum vor allem in der Schweine- und Geflügelmast Verwendung, wird aber auch in Form von Grünfutter, Heu und Silage für die Fütterung von Wiederkäuern genutzt. In der industriellen Nutzung liegt der Fokus auf der Ethanol-, Papier- und Besenherstellung (Berenji und Dahlberg 2004).

Neben der Nutzung als Futtermittel eröffnet die Ganzpflanzennutzung auch energetische Verwertungspfade: Die einjährige Pflanze kann dabei zur Gewinnung von Ethanol aus den Körnern oder bei Zuckertypen aus dem safthaltigen Stängel sowie als Biogassubstrat (Ganzpflanzensilage) genutzt werden. Die Sorghum-Ganzpflanzensilage stellt mit Methanausbeuten von etwa 300–360 Nl CH_4/kg oTS ein sehr gut geeignetes Substrat für Biogasanlagen dar (Diepenbrock 2014). In Europa liegt der Fokus auf der Nutzung als nachwachsender Rohstoff in Biogasanlagen. Neben der Aufnahme in die Fruchtfolge als alternative Energiepflanze bieten die positive Wirkung als Pollentrachtpflanze für Bienen und die Resistenz gegen den Maiswurzelbohrer, der im intensiven Maisanbau zu erheblichen Ertragsverlusten führen kann, weitere ökologische Vorteile (Fora et al. 2013; Siede et al. 2021). Besonders unter trockenen Standortbedingungen zeigt Sorghum Vorteile, weil Wachstumsverzögerungen durch spätere Niederschläge teilweise besser ausgeglichen werden können als bei Silomais. Demgegenüber können eine langsamere Jugendentwicklung, spätere Saattermine und ein höherer Wärmebedarf die Ertragsbildung begrenzen und damit nachteilig wirken (Yu et al. 2004).

2.4 Hybridzüchtung von Sorghum

Die Einführung der Hybridzüchtung leistete einen entscheidenden Beitrag zur Ertragsverbesserung von Sorghum (Doggett 1988). Heterosiseffekte wurden in Sorghum erstmals in den 1920er-Jahren experimentell nachgewiesen (Conner & Karper 1927). Eine praktische Umsetzung dieser Erkenntnisse in der Züchtung erfolgte jedoch erst deutlich später. Der Grundstein für die Hybridzüchtung wurde 1954 mit der Entdeckung eines cytoplasmatisch männlichen Sterilitäts-Systems (CMS), dem A1(milo)-Cytoplasma, gelegt. Diese Entdeckung lieferte einen entscheidenden Impuls für die großflächige Hybridproduktion, da sie eine effiziente Saatgutproduktion ermöglichte (Stephens und Holland 1954). Daraufhin begann in den Vereinigten Staaten Mitte der 1950er-Jahre die kommerzielle Nutzung von Sorghum-Hybriden (Doggett 1988). Der Einsatz von Hybridsorten wird als wesentlicher Faktor für die Ertragssteigerung im Sorghumanbau angesehen. Schätzungen zufolge liegt die weltweite Produktion etwa 19 % höher, als dies ohne Hybridzüchtung der Fall wäre (Duvick 1999). In einzelnen Fällen zeigten sich bei den frühen Hybriden Ertragsvorteile bis zu 40 % (Klein et al. 2008). Neben dem A1-Cytoplasma wurden weitere CMS-Systeme entdeckt. Dazu gehören A2, A3, A4, Indian A4, A5, A6, 9E und die KS-Cytoplasmen (Webster und Singh 1964; Ross und Hackerott 1972; Worstell et al. 1984; Rakshit und Wang 2017). Trotz gleichwertig effizienter Alternativen wie dem A2-Cytoplasma findet bis heute vorrangig das A1-Cytoplasma in der kommerziellen Hybridzüchtung Verwendung (Schertz und Ritchey 1978; Reddy et al. 2007). Dies lässt sich durch den höheren Anteil an Restorern mit vollständiger Wiederherstellung der Pollenfertilität beim A1-Cytoplasma im Vergleich zu den anderen verfügbaren CMS-Systemen erklären (Reddy und Stenhouse 1994).

Durch die Verwendung eines CMS-Systems kann die Pollensterilität der Mutterlinie gewährleistet werden, sodass bei der Saatgutproduktion der Hybride die Bestäubung gezielt über die Vaterlinie erfolgt und daraus pollenfertiles F1-Saatgut hervorgeht. Da Sorghum überwiegend generativ genutzt wird und die Rispe eine zentrale Ertragskomponente darstellt, ist die Erzeugung pollenfertiler Hybride notwendig. Dies gelingt durch die Verwendung eines geeigneten Restorers als Pollenspender. Die Fähigkeit zur Wiederherstellung der männlichen Fertilität („Restoration“) in F1-Hybriden wird durch die im Zellkern des Restorers lokalisierten Rf-Gene determiniert. Restorer führen an den entsprechenden Loci dominante Allele, während Maintainerlinien, die der Erhaltung der CMS-Mutterlinie dienen, rezessive rf-Allele tragen. Aufgrund eines „normalen“ Cytoplasmas sind die Maintainer pollenfertil. Sowohl für

die Vermehrung der CMS-Komponente als auch für die Produktion pollenfertilen Hybridsaatguts wird jeweils dieselbe CMS-Komponente genutzt. Charakteristisch für die CMS-Komponente ist die Kombination eines männliche Sterilität induzierenden Cytoplasmas mit rezessiven rf-Allelen im Zellkern, die die männliche Sterilität erhalten (Abb. 4) (Becker 2019).

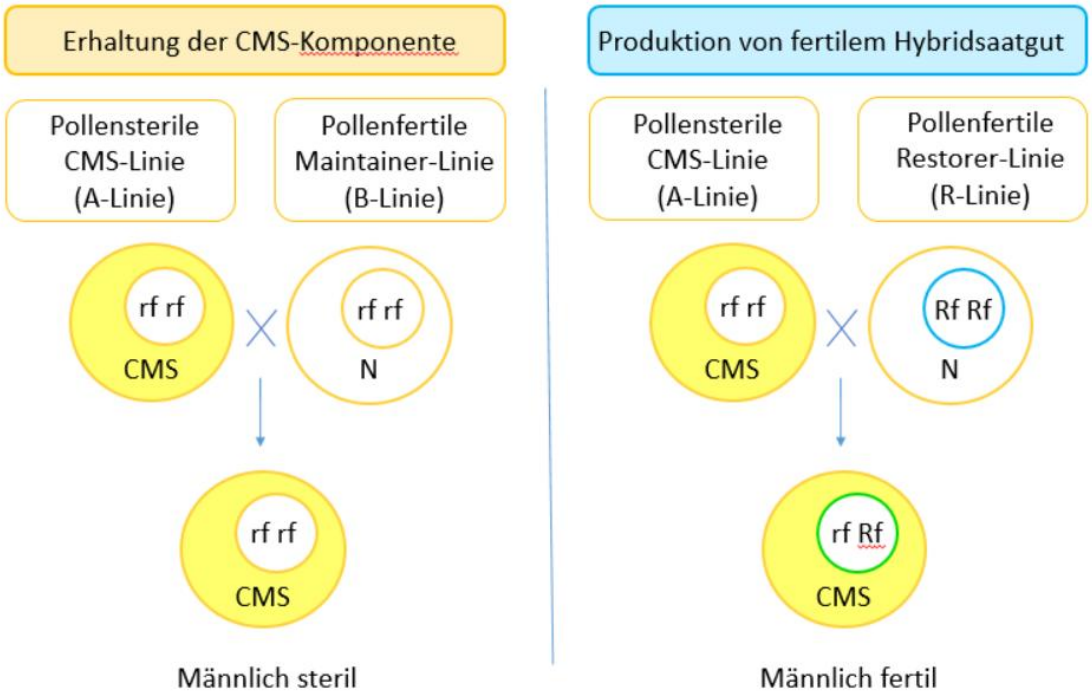

Abbildung 4: CMS-System zur Produktion von Hybridsaatgut, modifiziert nach Becker et al. (2019). Links: Erhaltung der CMS-Komponente. Die pollensterile CMS-Linie (A-Linie, CMS, rf rf) wird mit der pollenfertilen Maintainer-Linie (B-Linie, normales Cytoplasma N, rf rf) gekreuzt. Da keine Restorer-Allele vorhanden sind, bleibt die zytoplasmatisch bedingte männliche Sterilität erhalten. Rechts: Produktion von fruchtbaren Hybridsamen. Die pollensterile CMS-Linie (A-Linie, CMS, rf rf) wird mit der pollenfertilen Restorerlinie (R-Linie, normales Cytoplasma N, Rf Rf) gekreuzt. Die dominanten nuklearen Restorer-Gene (Rf) stellen im F1-Hybriden (rf Rf) die männliche Fruchtbarkeit wieder her, sodass pollenfertile und fruchtbare Hybridsamen entstehen.

Um die Mehrleistung von Hybriden gegenüber ihren Eltern bestmöglich auszuschöpfen, werden zunächst homozygote Inzuchtlinien generiert, die sich genetisch möglichst stark unterscheiden (Schnell 1982). Dabei sollten die wichtigsten agronomischen Merkmale bereits in beiden Elternlinien auf einem guten Niveau vorliegen (Link 1996). Die Auswahl geeigneter Elternkombinationen erfolgt anschließend über Kombinationstests, in denen sowohl die allgemeine Kombinationsfähigkeit (general combining ability, GCA) als auch die spezifische Kombinationseignung (specific combining ability, SCA) geprüft werden. Die Mehrleistung der Hybride im Vergleich zum Elternmittel wird als Heterosis bezeichnet (Becker 2019).

In Abhängigkeit von der Nutzungsform stehen bei der Züchtung unterschiedliche Zielmerkmale im Vordergrund. Der weltweit am häufigsten angebaute Sorghumtyp ist Kornsorghum. Hierbei ist das Erreichen einer reduzierten Pflanzenhöhe entscheidend, damit sich das Korn gut im Mähdrusch ernten lässt (Berenji und Dahlberg 2004). Für die Nutzung als Biogassubstrat werden vorrangig zwei Typen diskutiert: Der klassische „Biomasse-Typ" zeichnet sich typischerweise durch sehr hohe Wuchshöhen bis etwa 4 m, eine späte Blüte sowie vergleichsweise geringe Stärke- und Methangehalte aus. Demgegenüber kann der „dual-use-Typ" unter günstigen Bedingungen höhere Methanerträge je Flächeneinheit erzielen, was vor allem mit einer größeren Rispe und einem stärkeren Kornansatz bei Wuchshöhen bis rund 2,5 m zusammenhängt. Durch die geringere Wuchshöhe ist die Neigung zum Lager verringert. Perspektivisch stellt sich die Frage, ob der klassische „Biomasse-Typ" zugunsten eines kompakteren Ideotyps abgelöst werden sollte (Windpassinger et al. 2015).
In der Züchtung auf kältetolerante Hybriden erwies sich eine sehr strenge Vorauswahl potenzieller Elternlinien allein anhand ihrer mittleren Ausprägung der juvenilen Kältetoleranz als wenig zielführend. Als effizienterer Ansatz erwies sich das Durchführen von Tests auf die allgemeine Kombinationseignung (GCA, general combining ability), um passende Elternlinien zu identifizieren (Windpassinger und Justus Liebig University Giessen 2016). Für eine weitere Ausdehnung des Sorghumanbaus in gemäßigten Klimaten und zur Absicherung stabiler Erträge ist eine zusätzliche Verbesserung der juvenilen sowie der reproduktiven Kältetoleranz notwendig (Schaffasz et al. 2019a).

2.4.1 Heterosis

Heterosis bezeichnet die Mehrleistung einer aus zwei genetisch entfernten, homozygoten Inzuchtlinien entstandenen Hybride. Befruchtungssystem, Komplexität des Merkmals und die genetische Distanz der Elternlinien bestimmen das Ausmaß der Heterosis (Becker 2019). Sorghum ist primär ein Selbstbestäuber (Poehlman, 1987). So fällt die Heterosis bei Sorghum geringer aus als bei vorrangigen Fremdbefruchtern wie Mais, aber deutlich höher als bei überwiegenden Selbstbefruchtern wie Gerste (Duvick 1999; Becker 2019). Das Heterosisniveau kann in Abhängigkeit vom Merkmal variieren. Komplexe quantitative Merkmale weisen in der Regel eine höhere Heterosis auf als Merkmale mit geringerer Komplexität (Becker 2019). Um den Heterosiszuwachs zu maximieren, ist die Nutzung verschiedener heterotischer Pools mit einer möglichst weiten genetischen Distanz notwendig

(Schnell 1982). Dabei sollten die wichtigsten agronomischen Merkmale jedoch in beiden Eltern vorliegen (Link 1996). Die genetische Erklärung der Heterosis stützt sich auf drei mögliche Hypothesen: Überdominanz, Dominanz und Epistasie. Während die Überdominanzhypothese besagt, dass die heterozygote Form per se besser ist als die homozygoten Inzuchtlinien, erklärt die Hypothese der Dominanz die Heterosis durch die partielle oder dominante Vererbung des jeweils günstigeren Allels. Die Epistasiehypothese beschreibt nicht-additive Wechselwirkungen zwischen Allelen an verschiedenen Loci, welche für die Heterosis verantwortlich sind (Becker 2019).

Zur Bestimmung der Mehrleistung der Hybride werden häufig Mid-Parent-Heterosis (MPH) und Best-Parent-Heterosis (BPH) als Parameter herangezogen. Die MPH misst die Differenz der Leistung der Hybride im Vergleich zum Durchschnitt ihrer ingezüchteten Elternlinien, die BPH hingegen die Differenz der Leistung der Hybride zum besseren Elternteil. Während die MPH für quantitativ-genetische Analysen die relevante Kenngröße darstellt, ist im züchterischen Kontext die BPH von größerem Interesse (Kaeppler 2012; Schaffasz et al. 2019b).

Die Heterosis drückt sich bei *Sorghum bicolor* durch eine frühere Blüte, höhere Korn- und Biomasseproduktion sowie größere Stängel und Rispen aus. Durch die daraus resultierenden größeren Rispenköpfe erhöht sich der Druschanteil (Quinby 1963). Obwohl der Proteingehalt des Korns der Hybriden im Vergleich zu den Elternlinien geringer ist, resultiert aufgrund der insgesamt höheren Erträge ein größerer Rohproteinertrag pro Hektar (Kambal und Webster 1966). Die stärkste Ausprägung der Heterosis zeigt sich beim Kornertrag. Als das am engsten mit dem Kornertrag verknüpfte Merkmal erwies sich dabei die Kornzahl pro Rispe (Kirby und Atkins 1968). Selbst bei einem mit den Eltern vergleichbaren Panicle Harvest Index produzieren Hybriden eine höhere Anzahl an Körnern (Blum et al. 1990). Die Kornzahl pro Rispe stellt ein komplexes Merkmal dar, da sie durch mehrere Komponenten beeinflusst wird, darunter die Anzahl der Quirle pro Rispe, die Zahl der Äste je Quirl sowie die Anzahl der Körner pro Ast (Blum 1967). Bestockung, Anzahl der Blätter, Blattflächendurchmesser und Saccharosekonzentration zeigen bei Sorghum wenig bis keine Heterosis (Blum et al. 1990; Pfeiffer et al. 2010). Die ausbleibende Heterosis in Bezug auf die Saccharosekonzentration im Stängel, welche bei Zuckerhirse von großer Bedeutung ist, könnte auf eine erreichte physiologische Obergrenze in den Elternlinien durch wiederholte Selektion auf hohe

Saccharosekonzentrationen zurückzuführen sein (Pfeiffer et al. 2010). Hinsichtlich der juvenilen und reproduktiven Kältetoleranz von *Sorghum bicolor* zeigen sich die Hybriden toleranter gegenüber Kälte als ihre Elternlinien. Dies spricht in beiden Fällen für ein heterotisches Merkmal (Schaffasz et al. 2019b; Windpassinger 2016).

Für die Kältetoleranz im reproduktiven Stadium wurde zudem festgestellt, dass Hybriden aus zwei kälteempfindlichen Elternlinien eine stärkere Heterosis aufweisen als solche aus zwei kältetoleranten Eltern. Dies deutet auf ein quantitativ ausgeprägtes und dominant vererbtes Merkmal hin. Als möglicher Beitrag zur Mehrleistung der Hybriden wird unter anderem eine erhöhte Pollenproduktion infolge größerer Antheren diskutiert (Schaffasz et al. 2019b).

2.5 Empfindlichkeit gegenüber Kälte von *Sorghum bicolor*

Sorghum, als eine aus den Tropen stammende und dementsprechend an Wärme angepasste Kulturpflanze, zeigt eine erhöhte Empfindlichkeit gegenüber kühleren Temperaturen. Für die Ausweitung des Anbaus in die gemäßigten Breiten, stellt dies eine wesentliche Einschränkung dar. Bei *Sorghum bicolor* wird die Kälteempfindlichkeit in zwei kritische Entwicklungsabschnitte unterteilt: Zum einen in die Empfindlichkeit während des Jugendstadiums und zum anderen in die Empfindlichkeit in der frühen reproduktiven Phase (Maulana und Tesso 2013).

Das Jugendstadium ist durch rein vegetatives Wachstum gekennzeichnet, während mit der Ausbildung generativer Gewebe der Übergang in die reproduktive Entwicklungsphase erfolgt (Rutishauser 1969). Für die Kälteempfindlichkeit im juvenilen Stadium konnten bereits mehrere bedeutende Quantitative Trait Loci (QTL) identifiziert und entsprechende kältetolerante Genotypen selektiert werden (Thakur et al. 2010; Maulana und Tesso 2013; Parra-Londono et al. 2018; Schaffasz et al. 2019a). Eine vielfältige genetische Ressource für Kältetoleranz gegenüber niedrigen Temperaturen in der Keim- und Auflaufphase stellen die aus China stammenden „Kaoliang“-Landrassen dar (Franks et al. 2006).

Im Gegensatz zur juvenilen Kältetoleranz ist die Kältetoleranz im reproduktiven Stadium weniger gut erforscht, hat aber keineswegs geringere Auswirkungen. Kältestress in der reproduktiven Phase kann – je nach Schwere des Stresses und Empfindlichkeit des Genotyps – zu einer verringerten oder ausbleibenden Samenbildung und somit im Extremfall zu 100 %

Ertragsverlust führen (Maulana und Tesso 2013; Chopra et al. 2017; Emendack et al. 2021). Die bisherige Literatur betont vor allem die verringerte Pollenfertilität (Caddel und Weibel 1971; Downes und Marshall 1971) als den hauptlimitierenden Faktor für den Sorghumanbau in gemäßigten Klimazonen (Maulana und Tesso 2013). Brooking (1976) beschreibt die Leptotänphase sowie die Interphase der Mikrosporenzellen als die gegenüber Kälte empfindlichste Phase. Nach Kälteeinfluss in diesem Entwicklungsstadium konnten bei kälteempfindlichen Pflanzen neben einer verringerten Pollenfertilität auch ein Rückgang der Pollenzahl beobachtet werden (Brooking 1976; Sharma und Nayyar 2016). Im Widerspruch dazu konnte Schaffasz et al. (2019a) keinen Zusammenhang zwischen Polleneigenschaften und Ertragsparametern bei Sorghumhirse unter dem Einfluss von Kältestress feststellen. Im Einklang damit steht, dass auch bei Mais kein direkter Zusammenhang zwischen Pollenerzeugung und Kornertrag vorliegt (Westgate et al. 2003). Zusätzlich wird eine verminderte Fruchtbarkeit des weiblichen Blütenorgans, genauer gesagt die Rezeptivität des Pistils, als mögliche Ursache für den verminderten Kornansatz bei kälteempfindlichen Genotypen diskutiert (Osuna-Ortega et al. 2003; Thakur et al. 2010). Auch bei anderen Arten konnte eine erhöhte Empfindlichkeit des weiblichen Gametophyten gegenüber Kältestress beobachtet werden. So führt bei *Cryptantha flava* der Stress zu einem irreversiblen Abort der Eizelle (Casper 1990). Auch bei Kichererbsen wirkt sich Kälte negativ auf das weibliche Blütenorgan aus. Hier kommt es zu einer verzögerten Eizellenreifung und erhöhtem Abort des Embryos (Srinivasan et al. 1999).

Schaffasz et al. (2019b) konnten mehrere Zuchtlinien und Hybriden mit hoher Toleranz gegenüber Kälte im reproduktiven Entwicklungsstadium identifizieren. Hinsichtlich der Komplexität der Merkmale juvenile und reproduktive Kältetoleranz kann angenommen werden, dass es sich bei beiden um heterotische und quantitative Merkmale handelt (Schaffasz et al. 2019a; Schaffasz et al. 2019b; Windpassinger 2016; Osuna-Ortega et al. 2002). Gene, die an der reproduktiven Kältetoleranz beteiligt sind, werden dominant vererbt (Schaffasz et al. 2019b). Eine additiv vererbte QTL-Kältetoleranz in der Reproduktionsphase ist ebenfalls bei Reis beschrieben (Suh et al. 2010). Eine Korrelation zwischen juveniler und reproduktiver Kältetoleranz wurde nicht gefunden (Chakrabarty et al. 2021).

2.5.1 Phytohormonreaktion auf Kältestress

Die Phytohormonreaktionen auf Kältestress bei *Sorghum bicolor* sind bisher wenig erforscht. Bei anderen Gräsern wurde Jasmonsäure (JA) als ein beteiligtes Phytohormon identifiziert, das in der Entwicklung von generativem Gewebe eine wichtige Rolle spielt (Cai et al. 2014). Abscisinsäure (ABA) und Gibberellinsäure (GA) sind ebenfalls an der Bildung generativen Gewebes beteiligte Phytohormone. Kühle Temperaturen von < 20 °C in der reproduktiven Phase haben bei Reis *Oryza sativa* einen Einfluss auf den Stoffwechsel. Durch den veränderten Stoffwechsel kommt es zu einer Beeinträchtigung bei der Bildung fertiler Pollen (Thakur et al. 2010). Durch die geringeren Temperaturen nehmen die Enzymaktivität sowie die Reaktionsrate ab, und der Stoffwechsel wird neu konfiguriert. Die ABA-Konzentration steigt an, was zu einer Unterdrückung des tapetumspezifischen Invertasegens OCINV4 im frühen Mikrosporenstadium führt. Dadurch wird die Synthese des Enzyms tapetumspezifische Invertase (OCINV4) unterbunden, das für die Spaltung von Saccharose in Glucose und Fructose zuständig ist. Die ausbleibende Spaltung führt zu einer Erhöhung der Saccharosekonzentration, während die Glucosezufuhr zum Tapetum und zu den Pollen blockiert ist. Durch die ausbleibende Zufuhr von Zucker kann das Tapetum nicht abgebaut werden. Es kommt zu einem irreversiblen Prozess, der Hypertrophie, der sterile Pollen verursacht (Thakur et al. 2010; Parish et al. 2012; Sharma und Nayyar 2016; Zhu et al. 2015). Die Fähigkeit der Antheren, unter Kältestress lebensfähige Pollen zu entwickeln, hängt davon ab, ob die Gensequenzen (OSZEP1 und OSNCED3) vorhanden sind, um eine niedrigere ABA-Konzentration aufrechtzuerhalten und einen ausreichenden Pool an bioaktiven Gibberellinen zu akkumulieren. Die identifizierten kältetoleranten Reis-Genotypen synthetisieren nicht nur geringere Mengen an ABA, sondern weisen gleichzeitig einen erhöhten ABA-Katabolismus durch eine erhöhte Expression von ABA-Hydroxylierungsgenen auf (Oliver et al. 2007; Ji et al. 2011; Parish et al. 2012; Sharma und Nayyar 2016). Zudem wird bei kältetoleranten Reispflanzen eine erhöhte ABA-Abbaurate durch Konjugation oder Oxidation diskutiert, um die ABA-Konzentration zu senken. Dabei ist die Expression beider ABA-8-Hydroxylase-Gene, ABA8ox1 und ABA8ox2, bei kältetoleranten Genotypen höher (Nambara und Marion-Poll 2005; Oliver et al. 2007). In Bezug auf das Aufrechterhalten eines ausreichend hohen Pools an Gibberellinen wurde bei Reispflanzen ein veränderter Biosyntheseweg von GA4 und GA7 identifiziert (Sharma und Nayyar 2016). Infolge erhöhter ABA-Werte und unzureichender Pools bioaktiver Gibberelline bei fehlender Kältetoleranz werden Probleme mit der

Hypertrophie tapetaler Zellen und abnormalem Pollenschlauchwachstum beschrieben. Die Unterdrückung des für zellwandgebundene saure Invertase codierenden Gens sowie des Monosaccharid-Transporter-Gens stört den Zuckertransport zum Tapetum, was zu abnormaler Pollenentwicklung und reduzierter Kornzahl führt (Oliver et al. 2005; Mamun et al. 2006).

Neben den genannten Phytohormonen könnten auch Auxin und Jasmonsäure eine entscheidende Rolle bei der Bildung generativen Gewebes von *Sorghum bicolor* spielen. An *Arabidopsis thaliana* wurde gezeigt, dass der Auxintransport die unabhängige Regulierung der Filamentverlängerung beeinflusst, während das in den Antheren synthetisierte Auxin eine wichtige Rolle bei der Koordination der Antherendehiszenz und der Pollenreifung spielt (Cecchetti et al. 2008). Jasmonsäure und ihrer biologisch aktiven Form Jasmonsäure-Isoleucin wird ebenfalls eine Schlüsselrolle im Zusammenhang mit der Beteiligung von Phytohormonen an der Kältetoleranz bei verschiedenen Poaceae-Arten zugeschrieben (Du et al. 2013; Cai et al. 2014). In *Arabidopsis* wurde gezeigt, dass die JA-Biosynthese die Expression kälteinduzierter Gene über den CBF-Transkriptionsweg reguliert, die für eine verbesserte Kältetoleranz verantwortlich sind (Hu et al. 2017).

2.6 Ziel dieser Arbeit

Sorghum bicolor ist eine vielseitige Kulturpflanze, die weltweit in zahlreichen Klimazonen angebaut wird. In Europa, und insbesondere auch in Deutschland, spielt der Anbau bislang eine untergeordnete Rolle. Vor dem Hintergrund zunehmender Klimaextreme und des Bedarfs an ressourceneffizienten Alternativen zu Mais gewinnen alternative Kulturarten zunehmend an Bedeutung.

Die vorliegende Arbeit verfolgt das Ziel, ein breiteres Verständnis der reproduktiven Kältetoleranz bei Sorghum zu gewinnen, indem mehrere offene Fragen geklärt werden: (I) Welche Rolle spielen das Entwicklungsstadium von Sorghum und die Dauer des Kältestresses? (II) Lässt sich ein verringerter Kornansatz allein durch eine reduzierte Pollenfertilität erklären? Oder gibt es Hinweise auf eine stärkere Bedeutung der weiblichen Blütenorgane? (III) Lässt sich in reziproken Hybriden eine Dominanz maternaler oder paternaler Effekte der Kältetoleranz beobachten?

Ein weiteres Ziel stellt das Verstehen der physiologischen Mechanismen der reproduktiven Kältetoleranz bei Sorghum im Zusammenhang mit Phytohormonen dar, indem folgende offene Fragen adressiert werden: (I) Welche Phytohormone werden unter Kältestress herunter- bzw. hochreguliert? (II) Welche Unterschiede in der Phytohormonantwort bestehen zwischen kältetoleranten und kälteempfindlichen Genotypen? (III) Wie unterscheiden sich die Phytohormonspiegel zwischen Entwicklungsstadien während der frühen reproduktiven Phase? (IV) In welchem Umfang lassen sich diese Ergebnisse aus der vorhandenen Literatur zu verwandten Arten übertragen?

Insgesamt soll diese Arbeit dazu beitragen, die Ursachen und Mechanismen reproduktiver Kälteempfindlichkeit bei Sorghum präziser zu erfassen und daraus ableitbare Ansatzpunkte für die Züchtung zu liefern. Die Ergebnisse können somit die Entwicklung kältetoleranter Sorghum-Sorten mit höherer Ertragsstabilität unterstützen und die Etablierung dieser Kulturpflanze in gemäßigten Klimaräumen fördern.

3 Publikation I

Reproductive Cold Stress in Contrasting Sorghum Genotypes: Is Pollen Fertility Really the Crucial Trait?

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Reproductive Cold Stress in Contrasting Sorghum Genotypes: Is Pollen Fertility Really the Crucial Trait?

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ABSTRACT

The influence of cold stress during the reproductive phase can lead to substantial yield losses in sorghum. In order to extend cultivation into temperate regions, a better understanding of reproductive cold tolerance is essential for breeding progress. To further elucidate the mechanisms responsible for cold tolerance, a cold-tolerant and a cold-sensitive parental line, along with their reciprocal F1 hybrids, were subjected to cold stress at various stages of reproductive development, with a focus on pollen fertility and receptivity of female floral organs. For this purpose, pollen measurements were conducted using impedance flow cytometry, and the *panicle harvest index* was determined post-maturation. While existing literature primarily attributes reduced pollen fertility as the cause of decreased seed set, this study provides evidence that female floral organs might be more affected than previously assumed. We found that the onset of generative tissue formation until BBCH39 (flag leaf visible) is the most cold-sensitive developmental stage and that there is no predominance of maternal or paternal effects associated with the inheritance of cold tolerance in reciprocal F1 hybrids. These findings offer valuable insights for the development of cold-tolerant sorghum varieties to enable cultivation in colder regions and enhance yield stability in temperate climates. Further studies should aim at validating and expanding these findings from the limited number of representative genotypes analyzed in the present manuscript to global sorghum diversity.

1 | Background

The significance of cold tolerance in sorghum for expanding cultivation into temperate regions is attracting growing scientific and agricultural interest. As population density steadily increases, the availability of fertile soil decreases (Bindraban et al. 2012). Sorghum, one of the world's key cereals for food and feed production, is known for its adaptability to various environmental conditions (Doggett 1988; Berenji and Dahlberg 2004; Zheng et al. 2011; FAO 2024). Originating from semiarid regions of Africa, this crop (Rakshit and Wang 2016) demonstrates high drought tolerance, efficient water and nutrient utilization, resistance to *Diabrotica virigifera* (Oyediran et al. 2004), and the ability to thrive on marginal soils, providing a competitive advantage for future climate changes (Patil 2017). However, cold stress, particularly in temperate regions but also in tropical high-altitude environments, presents a significant challenge (Pinthus and Rosenblum 1961). Sorghum is not only sensitive in the seedling stage but also exhibits increased susceptibility to cold stress during the reproductive phase, resulting in reduced or even no seed set (i.e., complete yield loss), depending on the severity of the stress and susceptibility of the genotype (Maulana and Tesso 2013; Chopra et al. 2017; Emendack et al. 2021).

Existing literature predominantly emphasizes reduced pollen fertility (Caddel and Weibel 1971; Downes and Marshall 1971) as the primary limiting factor for sorghum cultivation in temperate regions (Maulana and Tesso 2013). However, the extent

to which the female floral organ, more precisely the pistil receptivity, is affected by cold stress is less well explored but has been a subject of discussion (Osuna-Ortega et al. 2003). If the stigma is unable to capture or properly transmit pollen under cold stress, fertilization cannot occur, leading to reduced or completely absent seed formation and severe yield losses, even when fertile pollen is present in sufficient quantities. Because the stigma plays a central role in successful fertilization, understanding its response to cold stress is essential for developing strategies to breed sorghum with improved cold tolerance. A detailed investigation of this aspect could help to establish targeted breeding approaches that consider not only pollen fertility but also stigma receptivity, thereby optimizing reproductive performance in colder climates. Considering the complexity of the traits, juvenile and reproductive cold tolerance can be assumed to be both heterotic and quantitative traits (Schaffasz, Windpassinger, Friedt, et al. 2019; Schaffasz, Windpassinger, Snowdon, et al. 2019; Windpassinger et al. 2015) Alleles involved in reproductive cold tolerance seem to be inherited dominantly, and a sustained breeding progress can be expected due to high phenotypic variation and high heritability (Schaffasz, Windpassinger, Snowdon, et al. 2019).

This work aims to gain a broader understanding of reproductive cold tolerance in sorghum, by unraveling several open questions: (i) What role do the developmental stage of sorghum and the duration of cold stress play; (ii) can reduced seed set be explained by reduced pollen fertility solely; (iii) or is there evidence for a more prominent role of female floral organs; (iv) can we observe a predominance of maternal or paternal cold tolerance effects in reciprocal hybrids.

Altogether, this study could contribute to speeding up the breeding progress toward cold tolerant sorghum with enhanced yield stability in temperate areas.

2 | Materials and Methods

2.1 | Plant Material

For the experiments, one cold-tolerant and one cold-sensitive inbred line and their reciprocal F1 hybrids were studied. The two inbred lines were selected from a diversity set of *Sorghum bicolor* ($n = 330$) consisting of inbred lines of different origin, utilization and subspecies. Traits such as seed yield and *panicle harvest index* (PHI) under cold conditions were used to evaluate reproductive cold tolerance (Chakrabarty et al. 2021).

Thus, genotype SB14011, a breeding line developed and selected under German conditions, was identified as cold tolerant and SC1056, a conversion line originating from Sudan, as cold sensitive. In addition, reciprocal F1 hybrids, shown in Figure 1, were created by hand emasculation. (Because both inbred lines are restorers in A1 cytoplasm, hand emasculation was the only way to create hybrids among them.)

2.2 | Defining Growth Stages Based on BBCH Scale

To examine the plants at different developmental stages, the BBCH scale was used, a standardized, detailed coding system for the phenological developmental stages of monocotyledons and dicotyledons. This scale is based on a decimal system, divided into macrostages and microstages. Its structure follows the grain scale developed by Zadoks et al. (1974) in order to avoid major changes to an already well-established and widely used scale. A graphical overview of the sorghum growth stages is provided in Figure 2a. In the present work, the plants were examined for the influence of cold from BBCH32, BBCH35, BBCH39, and BBCH51 onwards (Figure 2b). At BBCH32, the plant is in the two-node stage; i.e., the second node is perceptible, at least 2 cm from the first node. At BBCH35, the plant has reached the

FIGURE 1 | Cold-tolerant parental line SB14011 and cold-sensitive parental line SC1056 and their reciprocal F1 hybrids (SB×SC and SC×SB) (grown under optimal conditions [see Table 1]).

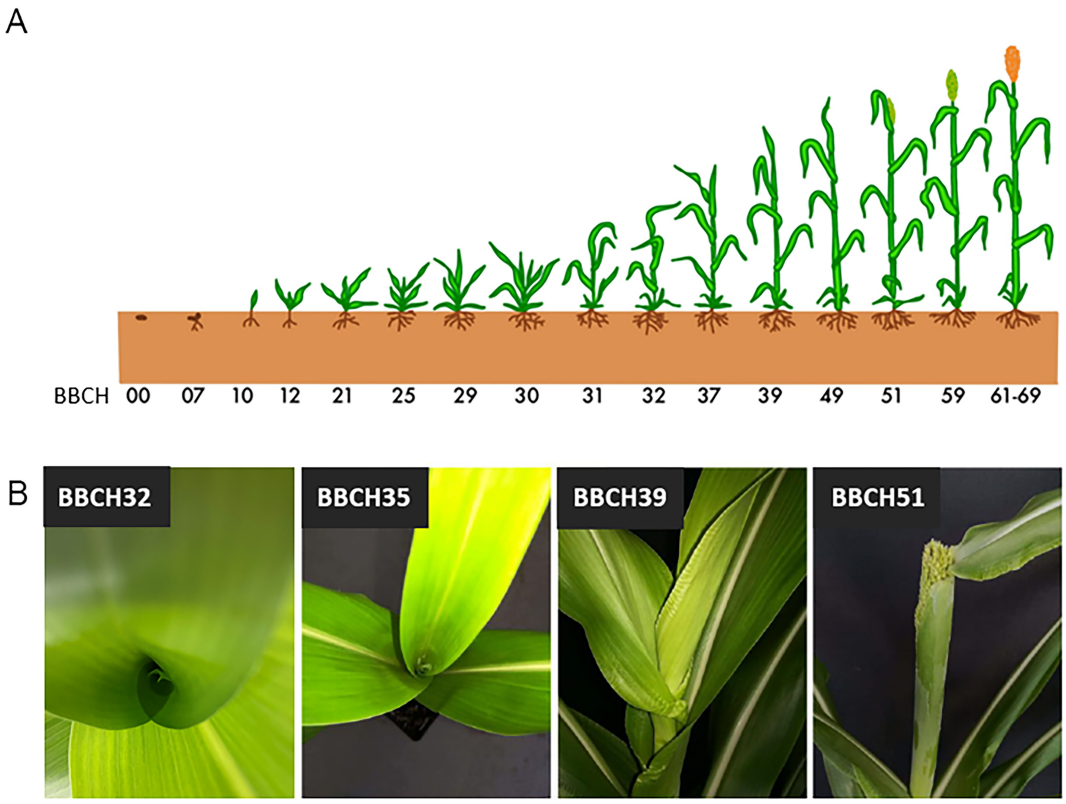

FIGURE 2 | (a) BBCH scale for sorghum in reference to the cereal scale developed by Zadoks et al. (1974). (b) Overview of the developmental stages to be examined—BBCH32: flag leaf not yet visible, seat low down; BBCH35: flag leaf not yet visible, shortly before the appearance of the leaf tip; BBCH39: ligule of the flag leaf visible; BBCH51: emergence of the panicle.

five-node stage; i.e., the fifth node is at least 2 cm from the fourth node. The flag leaf is very small at this stage, has its seat relatively far down, and is completely curled up. At stage BBCH39, the flag leaf is now fully developed and has visible ligules. At BBCH51, heading has begun; i.e., the panicle tip emerges from the leaf sheath.

2.3 | Climate Chamber Experiments

The climate chamber experiment was carried out at the IFZ Research Center for Biosystems at the Justus Liebig University in Giessen, Germany. Both inbred lines and their reciprocal F1 hybrids were tested under controlled cold stress and optimum growth conditions at different stages (BBCH32, BBCH35, BBCH39, and BBCH51) in the reproductive phase. Plants were grown in 17×17×17 cm pots filled with Fruhstorfer type N soil and were watered and fertilized following good horticultural practice to exclude any other stresses. During emergence and early vegetative growth, all plants were grown under optimum conditions (30°C/13 h during the day and 24°C/11 h at night, with a relative humidity of 60% and metal halide lamps for lighting). For each of the above-mentioned BBCH stages, randomly selected plants were transferred to a cold stress chamber (25°C/13 h during the day and 7°C/11 h at night, with relative humidity of 60% and LED lighting), where they remained from the respective BBCH stage until the beginning of the grain filling phase (BBCH 71). By this approach, the following two factors are combined: (i) a specific initial phase and (ii) different stress durations.

For each genotype and BBCH stage, three plants were stressed. These individual plants were considered as replicates for statistical evaluation.

2.3.1 | Male Versus Female Floral Organ

Transparent Cryovac bags (330 mm×750 mm, 15 µm) (Sealed Air, Charlotte, NC, USA) were placed over the panicles before flowering to ensure self-pollination for the considered seed set traits.

To analyze the effect of cross-pollination, a plant grown under optimal conditions ("male plant") was packed under the Cryovac bags together with a stressed plant ("female plant") (25°C/13 h during the day and 7°C/11 h at night, with relative humidity of 60% and LED lighting) of the same genotype shortly before anther emergence throughout the flowering period (Figure 3). Thus, when evaluating the receptivity of the pistil, it can be excluded that sterile pollen led to low PHI. The "female plants" were each stressed at the BBCH stages described above. This experiment was conducted for both inbred lines (susceptible line SC1056 and tolerant line SB14011) and their reciprocal hybrids (SB×SC and SC×SB), in three replicates each.

FIGURE 3 | Left: Pollen fertility experiment—individual sorghum plant bagged prior to anthesis to ensure self-pollination; right: Pollen receptivity experiment—stressed sorghum plant ("female plant") bagged together with an unstressed pollinator plant ("male plant") of the same genotype.

2.4 | Pollen Analysis via Amphasys Impedance Flow Cytometry (IFC)

Pollen analyses was conducted using IFC, manufactured by Amphasys AG (Amphasys AG, Root, Switzerland). This technique measures electrical capacity (and hence viability) of a cell, utilizing a small microfluidic chip where the pollen grains flow through and the electric charge of the cells is measured via different radio frequencies (Heidmann et al. 2016). Sample collection and measurements were carried out applying established protocols for sorghum described previously by Schaffasz, Windpassinger, Snowdon, et al. 2019.

2.5 | PHI

To determine the PHI (Krishnamurthy et al. 2014), at seed maturity the panicles were harvested by cutting them just below the first branches and dried. The total weight of the panicle was then determined before threshing and the pure seed weight after threshing.

Subsequently, the PHI was calculated based on the previously collected data using the following formula (Krishnamurthy et al. 2014):

$$\text{PHI} = \text{grain dry weight (i. e., seed yield per panicle)} / \text{panicle dry weight (before threshing)}$$

Consequently, a PHI value of 0 implies absolutely no seed set, while values close to 1 indicate a high seed set. However, even assuming complete spikelet fertility, PHI will be <1, due to the panicle raw weight. In addition, seed number was measured using seed-counter Contador (Pfeuffer GmbH, Kitzingen, Germany). All these traits (PHI and seed number) were scored on both stressed and nonstressed plants.

2.6 | Statistical Analysis

A multifactorial ANOVA was performed for all scored traits, taking into account the independent variables "treatment" and "genotype" as fixed factors to analyze possible interaction effects.

Hence, the ANOVA model applied here followed the general form:

$$Y = \mu + \text{Treatment} + \text{Genotype} + \text{Treatment} \times \text{Genotype} + \text{Error}$$

where Y represents the dependent variable, μ is the overall mean, "Treatment" and "Genotype" are fixed main effects, and "Treatment × Genotype" represents the interaction between these factors. The error term accounts for the random variation within the data (residual variance).

Separate one-way ANOVA analyses were performed for each specific trait to asses possible differences between treatment levels (BBCH32, BBCH35, BBCH39, BBCH51, and control) within a genotype. The independent variable in each analysis was "treatment", and the dependent variable was the respective trait. These characteristics included the yield parameters PHI and seed yield as well as the pollen parameters percentage of fertile pollen (%), cell concentration (cells/mL), and total number of fertile pollen (fertile pollen/mL) measured with the impedance flow cytometer.

Following each ANOVA, a least significant difference (LSD) post hoc analysis was performed to determine which treatments showed statistically significant differences from each other.

t-tests were performed to analyze significant differences between cross-pollination and self-pollination at a specific

treatment level. A paired *t*-test was performed to analyze significant differences between the parental lines SB14011 and SC1056 and their reciprocal F1 hybrids within a given treatment level.

Pearson's correlation coefficients were used to assess the relationship between the pollen traits and the yield parameters.

The statistical analysis and the presentation of the results were carried out in the R version 4.0.5.

3 | Results

3.1 | Sensitivity to Cold During the Reproductive Stage

3.1.1 | Yield Parameters

The impacts of cold stress varied significantly depending on the treatment stage and genotype (Figure 4). The cold-sensitive inbred line SC1056 exhibited a pronounced response to cold stress at BBCH32, BBCH35, and BBCH39. Both the PHI and seed yield significantly deviated from the control in this case. Upon closer examination of seed yield, a significantly lower seed yield was also observed after cold stress at BBCH51 compared with the control. In contrast, the cold-tolerant inbred line SB14011 showed neither significant differences in seed yield nor in PHI among the different treatments (Table 1).

The mean values of PHI and seed yield of genotype SC1056 were lowest after cold stress in the early reproductive stage (BBCH32, BBCH35, and BBCH39). For interpreting the results, it is important to highlight that the size of the panicle was also affected by cold stress (Figure 5).

Analyzing the impact of stress treatments on the reciprocal F1 hybrids, fewer distinctions were noted compared with the inbred lines. Only the hybrid SC×SB displayed a significantly reduced seed yield following exposure to cold stress at BBCH32, BBCH35, and BBCH39 (Figure 6), while for hybrid SB×SC, the stress treatments did not produce significant effects.

3.1.2 | Pollen Traits

Significant differences among various treatment levels within each genotype were found for nearly all parameters when

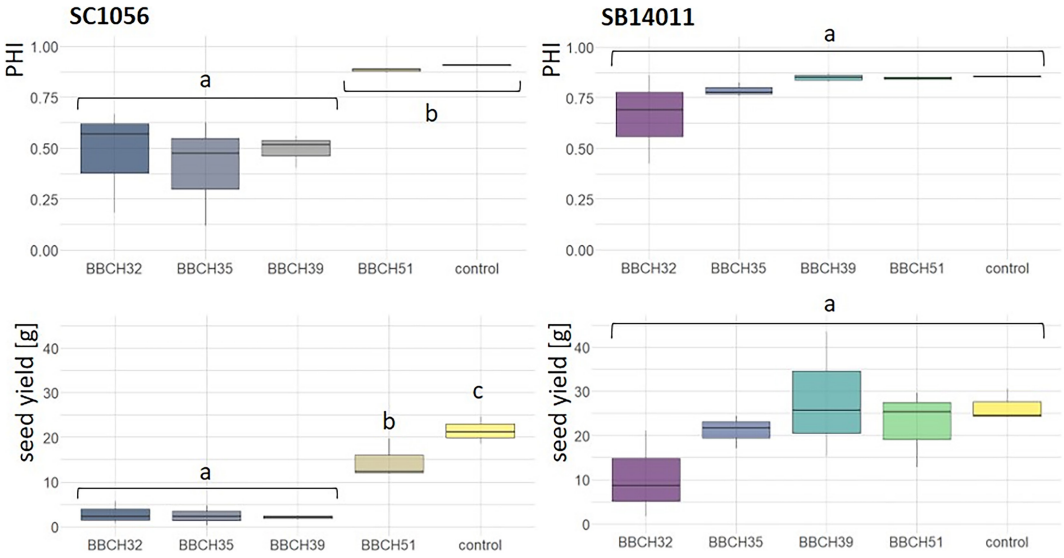

FIGURE 4 | Box plots depicting the panicle harvest index (PHI) and seed yield of a cold-tolerant (SB14011) and a cold-sensitive (SC1056) sorghum inbred line subjected to cold stress at developmental stages BBCH32, BBCH35, BBCH39, and BBCH51 and under optimal conditions (control); *p*-values in the Supporting Information (Table S2).

TABLE 1 | ANOVA (see M and M 2.6) analyzing possible effects of the different stress treatments and descriptive statistics for both inbred lines and their reciprocal F1 hybrids regarding the traits panicle harvest index (PHI) and seed yield.

		PHI					Seed yield (g)				
	df	MS	Error	Mean	Min	Max	MS	Error	Mean	Min	Max
SB14011	4	0.021	0.001	0.799	0.425	0.870	143.24	80.78	21.74	1.64	43.48
SB×SC	4	0.045	0.036	0.769	0.227	0.903	127.71	88.46	18.79	0.54	38.88
SC1056	4	0.176**	0.028	0.632	0.120	0.909	237.31***	7.98	8.68	0.22	24.60
SC×SB	4	0.032	0.019	0.800	0.315	0.899	291.29**	38.47	18.81	0.80	38

Significance level: ***0.001; **0.01; *0.05.

FIGURE 5 | Panicles of the cold-sensitive genotype (SC1056) and the cold tolerant genotype (SB14011) after cold stress from BBCH32, BBCH35 BBCH39, and BBCH51 and under control conditions.

analyzing pollen characteristics. The only exception was the proportion of fertile pollen, where no significant differences were observed between the treatments in the SC×SB hybrid. Particularly remarkable were the substantial variations in the proportion of fertile pollen between the SC1056 genotype and the F1 hybrid SC×SB, with values ranging from approximately 3% to 93% across all treatments (Table 2).

Upon examination of the pollen characteristics in Figure 7, it became evident that the proportion of fertile pollen significantly differed from the control after cold stress at BBCH32 and BBCH51 in the cold-tolerant genotype SB14011. In the hybrid SB×SC and the cold-sensitive genotype SC1056, only the proportion of fertile pollen after cold stress at BBCH51 and BBCH35, respectively, differed from the control. In the SC×SB hybrid, no differences were observed between the stress treatments and the control. When cell concentration and the total number of fertile pollen were considered, significant differences were observed between various treatments and the control in all genotypes. After cold stress at BBCH32, the two reciprocal hybrids exhibited higher cell concentration and a greater total number of fertile pollen compared with the control. The same phenomenon was observed in the two parental lines after cold stress at BBCH35. SB14011 additionally showed higher cell concentration and a greater total number of fertile pollen than the control after cold stress at BBCH39. The SB×SC hybrid exhibited lower cell concentration compared with the control after cold stress at BBCH51, while the total number of fertile pollen remained at a similar level to the control.

3.1.3 | Genotype×Treatment Interaction

In the examination of yield parameters, it became evident that the ranking of individual treatments in terms of PHI and seed yield was quite similar across all genotypes (Figure 8). In terms of PHI, all genotypes generally exhibited the lowest values following cold stress at BBCH32 and BBCH35, with the control achieving the highest values. A notable increase was observed from BBCH39 onwards in all genotypes. The PHI after cold stress at BBCH51 was relatively consistent across all genotypes. For seed yield, a similar pattern emerged. These yield parameters underscored the substantial difference in the early reproductive stages (BBCH32, BBCH35, and BBCH39) of the cold-sensitive genotype SC1056 in comparison to the other genotypes. Additionally, it was noteworthy that the seed yield after

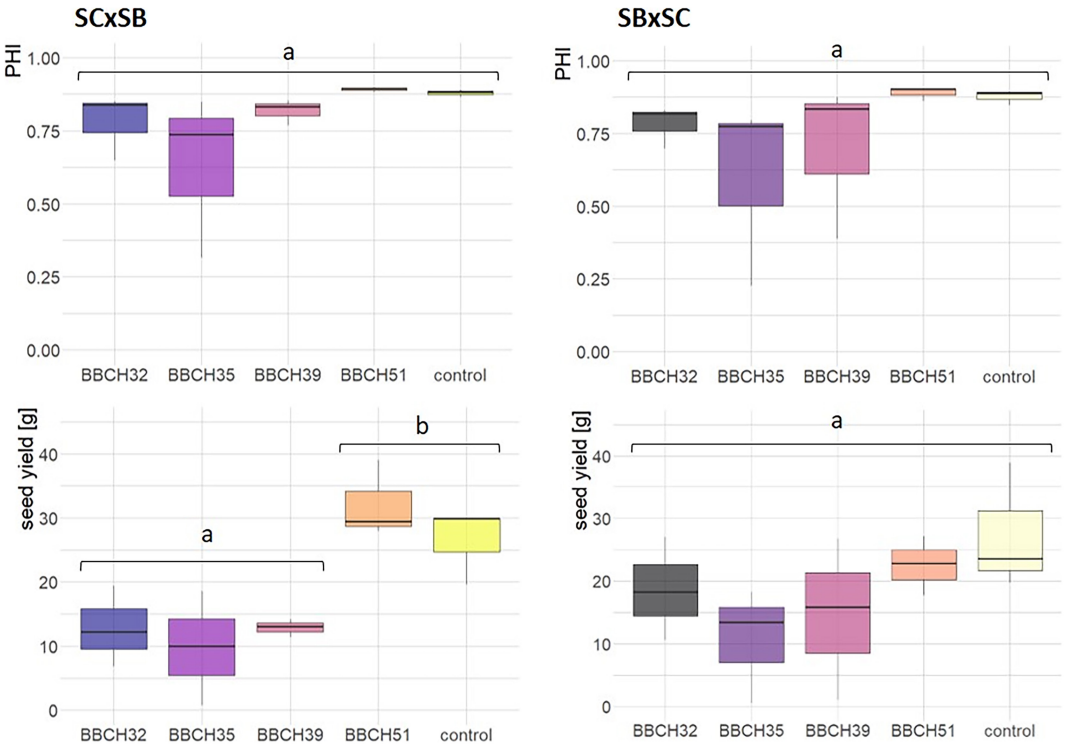

FIGURE 6 | Boxplots depicting the panicle harvest index (PHI) and seed yield of the reciprocal hybrids SC1056×SB14011 and SB14011×SC1056 subjected to cold stress at different developmental stages (BBCH32, BBCH35, BBCH39, and BBCH51) and under optimal conditions (control); *p*-values in the Supporting Information (Table S2).

cold stress at BBCH51 was significantly lower compared with the other genotypes and the control.

In summary, when assessing yield parameters, a consistent trend was observed across different genotypes with variations primarily dependent on the timing and duration of cold stress.

Figure 9 shows that the proportion of fertile pollen followed a similar ranking as observed in relation to the PHI. However, for the other two pollen traits, namely, cell concentration and the total number of fertile pollen, a somewhat different ranking became apparent, with noticeable differences between the two parental lines and their reciprocal hybrids. In the case of the cold-tolerant genotype SB14011, there was a significant increase in both traits after cold stress at BBCH35, while the cold-sensitive genotype SC1056 exhibited the lowest values. The two hybrids displayed a remarkably similar pattern concerning cell concentration and the total number of fertile pollen.

The cell concentration was higher the earlier the plants were exposed to cold stress. This is evidenced by the fact that the highest values were observed in plants that were subjected to cold stress from the BBCH32 stage, while the lowest values were observed in plants exposed to cold stress from the BBCH51 stage.

There was no significant genotype×treatment interaction concerning the two yield parameters, PHI and seed yield. In contrast, for all three pollen traits (proportion of fertile pollen, cell concentration, and total number of fertile pollen), a significant genotype×treatment interaction was observed (Table 3).

3.2 | Correlations Between Yield Parameters and Pollen Traits

Pearson's correlation was calculated to investigate the presence of any correlation between yield and pollen traits. The results are presented in Figure 10.

No significant correlation was observed between any of the collected yield parameters (PHI, panicle weight, and seed yield) and the pollen traits (cell concentration, proportion of fertile pollen, and cell count). As expected, the correlation between (total) panicle weight and seed yield was very high ($r=0.995$***), as well as between cell count and cell concentration ($r=0.903$***). Additionally, PHI displayed strong correlations with panicle weight (0.726***), seed yield (0.757***), and the proportion of fertile pollen with both cell count (0.446***) and cell concentration (0.441***).

3.3 | Parental Lines Versus Reciprocal F1 Hybrids

The *t*-test comparing genotypes within a certain treatment revealed significant differences after cold stress at BBCH35, BBCH39, and BBCH51, and in the control in terms of at least one of the two yield parameters. At BBCH32 and BBCH35, no significant differences in PHI were observed after cold stress (Figure S1). However, when considering grain yield, significant differences were found between the hybrid SB×SC after cold stress from BBCH32 and between the two parental lines after cold stress from BBCH35. While both parental lines significantly differ from each other in terms of PHI and grain yield after cold stress from BBCH39, the hybrid SC×SB only exhibits a significantly higher

TABLE 2 | ANOVA (see M and M 2.6) analyzing possible effects of the different stress treatments and descriptive statistics for both inbred lines and their reciprocal F1 hybrids regarding the traits proportion of fertile pollen (%), cell concentration (cells/mL), and total number of fertile pollen (cells/mL).

		Proportion of fertile pollen					Cell concentration					Total number of fertile pollen				
	df	MS	Error	Mean	Min	Max	MS	Error	Mean	Min	Max	MS	Error	Mean	Min	Max
SB14011	4	1194***	123	75.08	19.17	94.69	42 M***	2 M	3741	742	9782	29 M***	1.5 M	2885	142	8003
SB×SC	4	449.9**	115.6	77.52	41.12	94.62	23 M***	1.5 M	3432	1261	7356	14 M***	1.3 M	2707	625	5917
SC1056	4	2441.5**	255.4	70.58	3.37	92.99	12 M***	1.7 M	3031	4	6260	8.2 M***	1.4 M	2273	0.135	5480
SC×SB	4	400.4	186.2	74.64	3.55	92.68	9 M***	1.8 M	3247	20	7555	7.4 M***	1.4 M	2508	0.71	6830

Significance level: ***0.001; **0.01; *0.05.

PHI compared with the cold-sensitive parental line. After cold stress from BBCH51, the cold-tolerant parental line SB14011 significantly differs from all other genotypes in terms of PHI. When considering seed yield, only a significant difference is observed between the hybrid SC×SB and the cold-sensitive parental line SC1056. No significant differences in seed yield were observed in plants grown under control conditions. However, significant differences were found when comparing the two parental lines and when comparing the cold-tolerant parental line SC1056 with the hybrid SB×SC in terms of PHI. Figure 11 illustrates the differences in the yield parameter seed yield at different treatment stages. In addition to the significant differences, substantial variability within a genotype is also evident.

3.4 | Self-Pollination Versus Cross-Pollination

When comparing the two pollination methods, a significant difference was observed only in the PHI of cold-sensitive genotype SC1056 after cold stress at BBCH39 (Table 4). In terms of seed yield, only self-pollination and cross-pollination differed significantly after cold stress from BBCH35 of the cold-tolerant genotype SB14011. For all other genotypes and at all treatment stages, no differences in either PHI or seed yield were found between the two pollination methods. The same result was obtained in a one-way ANOVA pooling all genotypes and stress treatments.

Figure 12 illustrates that a significant difference due to pollination methods was only found for SC1056 at BBCH39. In the cold-sensitive inbred line SC1056, cross-pollination at that stage led to a markedly increased PHI, whereas in the cold-tolerant inbred line SB14011 at BBCH35, the response was the opposite, with cross-pollination apparently reducing the PHI (not significant).

Yet, it should to be acknowledged that after cold stress in BBCH32, BBCH35, and BBCH39, the scatter of the measured values in relation to PHI is high in some cases, regardless of pollination method. After cold stress in BBCH51, the PHI is consistently high for all genotypes, with a low scatter.

4 | Discussion

4.1 | Cold Sensitivity in the Reproductive Development

To identify the most cold-sensitive phases of reproductive development in sorghum, a cold-tolerant parental line (SB14011), a cold-sensitive parental line (SC1056), and their reciprocal F1 hybrids were exposed to cold stress from different stages of reproductive development onwards. The stages BBCH32, BBCH35, BBCH39, and BBCH51 were examined in detail. Using this experimental approach, the respective cold stress treatments differed not only in the timing of stress initiation but also in the duration of cold exposure, resulting in combined effects of these two factors.

When considering the PHI of the plants stressed at different developmental stages, the cold-sensitive genotype SC1056 was found to be most sensitive to cold stress after exposure in the early reproductive stages (BBCH32, BBCH35, and BBCH39). This confirms

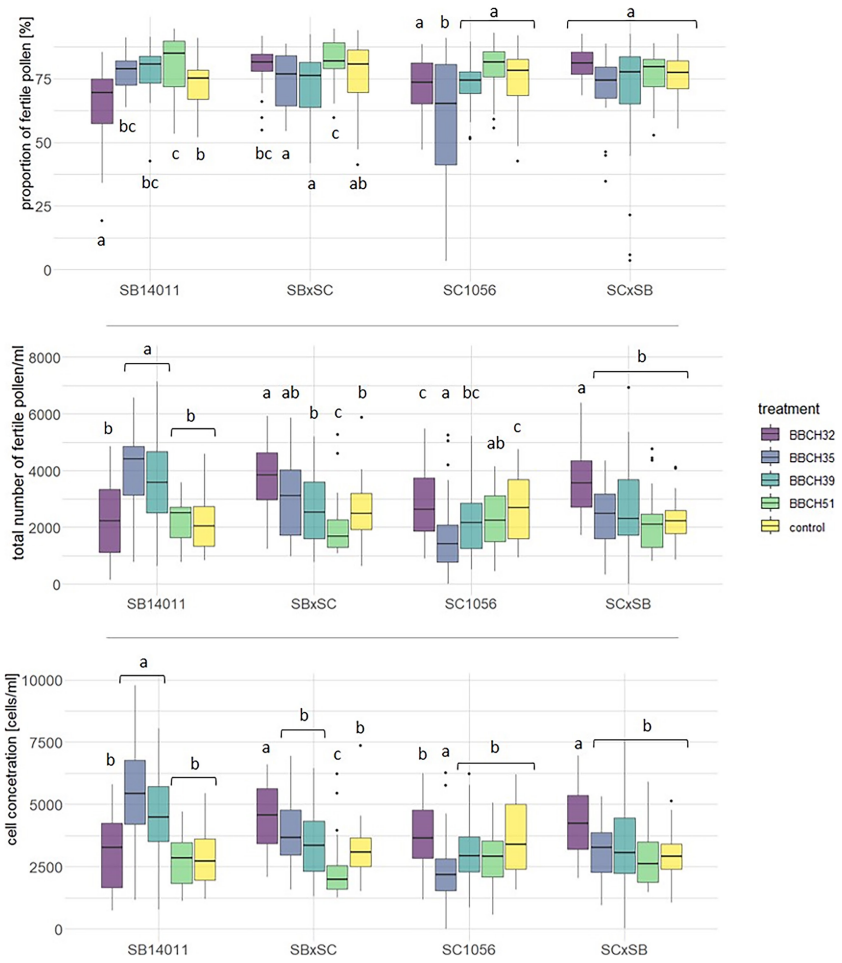

FIGURE 7 | Boxplots depicting the proportion of fertile pollen, cell concentration, and total number of fertile pollen of a cold-tolerant (SB14011), a cold-sensitive (SC1056) sorghum inbred line and their reciprocal F1 hybrids (SB×SC and SC×SB) subjected to the different treatments; *p*-values in the Supporting Information (Table S2).

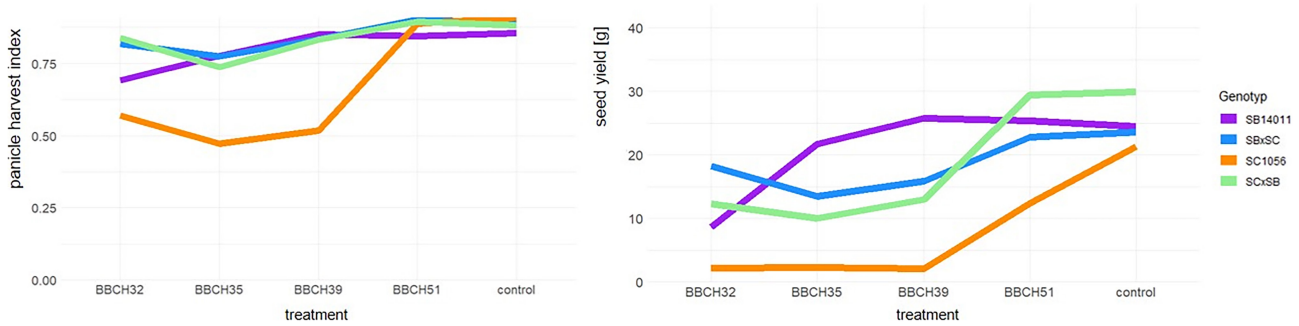

FIGURE 8 | Representation of the genotype×treatment interaction for the examined features panicle harvest index (PHI) and seed yield (g); Treatment means cold stress after BBCH32, BBCH35, BBCH39, and BBCH51 and under controlled conditions. Genotypes means the cold tolerant genotype SB14011 and the cold-sensitive genotype SC1056 as well as their reciprocal hybrids SB×SC and SC×SB.

the assumption made by Brooking (1976) that sorghum is most sensitive to cold before and during the leptotene stage or the interphase of microspore mother cells. After onset of panicle heading the sensitivity to cold decreases, although SC1056 still remains somewhat sensitive to cold temperatures at this stage. As shown in Figure 5, cold stress led to a reduction in panicle length, with earlier and prolonged stress exposure resulting in stronger effects. These findings highlight that not only the developmental stage of the plant but also the duration of cold stress plays a crucial role in the observed effects. While differences in panicle length are compensated for by the PHI, the reduction in grain yield is primarily attributable to smaller panicles.

In the test for existing genotype×treatment interactions, only the pollen traits showed significant results. The absence of interactions regarding the yield parameters (PHI and seed yield) can be explained by the similar response of the genotypes, underlining the strong effects of cold stress in early reproductive phase.

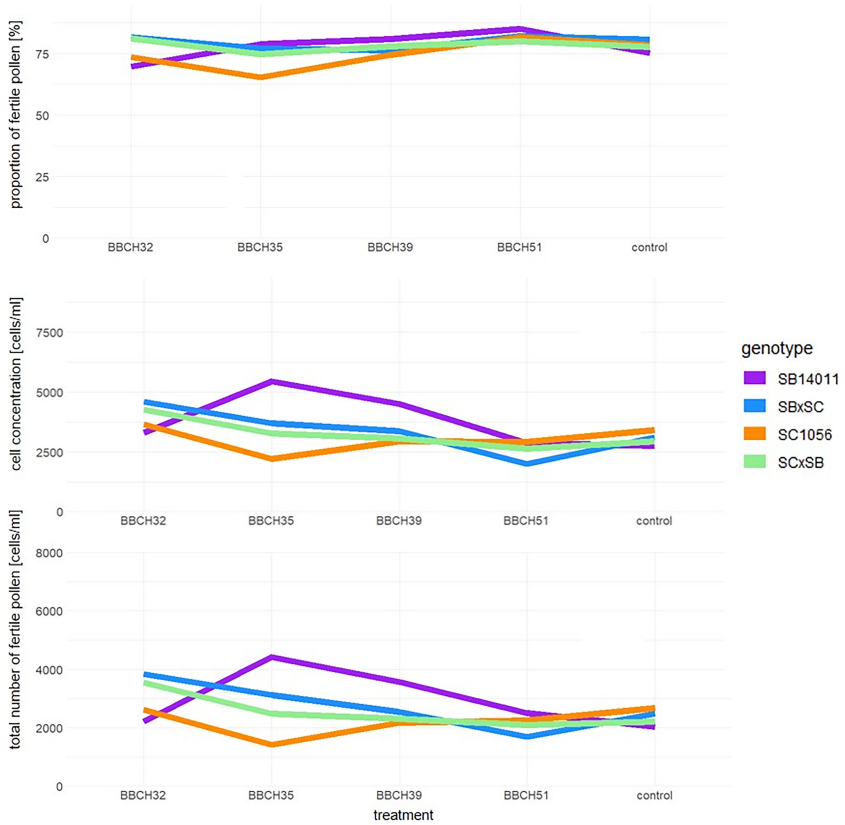

FIGURE 9 | Representation of the genotype × treatment interaction for the examined features proportion of fertile pollen (%), cell concentration (cells/mL), and total number of fertile pollen (cells/mL); treatment means cold stress after BBCH32, BBCH35, BBCH39, and BBCH51 and under controlled conditions. Genotypes means the cold tolerant genotype SB14011 and the cold-sensitive genotype SC1056 as well as their reciprocal hybrids SB × SC and SC × SB.

TABLE 3 | Results of a multifactorial ANOVA to investigate significant differences between treatments and genotypes, as well as genotype × treatment interactions, with respect to panicle harvest index (PHI), seed yield (g), proportion of fertile pollen (%), cell concentration (cells/mL), and total number of fertile pollen (cells/mL).

		PHI		Seed yield		Proportion of fertile pollen		Cell concentration		Total number of fertile pollen	
	df	ms	Error	ms	Error	ms	Error	ms	Error	ms	Error
Genotype	3	0.096*	0.023	490.2	53.9	0.1389***	0.017	14 M***	1.7 M	11 M***	1.4 M
Treatment	4	0.182***		549.2		0.1666***		26 M***		13 M***	
G × T	6	0.031		83.5		0.0939***		19 M***		15 M***	

Significance level: ***0.001; **0.01; *0.05.

Although not all genotypes exhibited significant yield losses after cold stress during the early reproductive phase (BBCH32, BBCH35, and BBCH39), the ranking of genotypes relative to each other remained unchanged. This suggests that both the timing of stress onset and its duration are critical factors influencing cold-induced yield losses.

4.2 | Importance of Pollen Traits for Reproductive Cold Tolerance

In the literature, a decrease in pollen quantity and fertility in response to low temperatures is commonly reported (Brooking 1976; Osuna-Ortega et al. 2003). However, the present study does not confirm this assumption. Only the cold-sensitive genotype SC1056 showed a reduced total number of fertile pollen after exposure to cold stress from BBCH35 onwards. For all other treatments and genotypes, yield reductions cannot be attributed to a decrease in fertile pollen quantity. One possible reason for this result being different in the present study may be the higher level of cold tolerance in the utilized germplasm compared with Brooking (1976) and Osuna-Ortega et al. (2003). Surprisingly, the SC × SB hybrid exhibited a higher cell concentration and an increased number of fertile pollen even after cold stress starting at BBCH32. Elevated cell concentration and total number of fertile pollen can also be observed in the cold-tolerant

line SB14011 after cold stress from BBCH35 and BBCH39, as well as in both F1 hybrids following cold stress from BBCH32. An increased production of fertile pollen in cold-tolerant plants, as opposed to susceptible ones, has already been observed (Sharma and Nayyar 2016). A possible explanation for the ability of sorghum to sustain the development of viable pollen under cold stress may lie in the presence of genes that accumulate a low concentration of ABA (abscisic acid), while simultaneously maintaining a sufficiently high pool of bioactive gibberellins. Additionally, reduced ABA synthesis could play a role, accompanied by a simultaneous increase in ABA catabolism due to enhanced expression of ABA hydroxylation genes. This mechanism has already been demonstrated in rice varieties (Sharma and Nayyar 2016; Parish et al. 2012). A finely tuned ABA regulation in the anthers or throughout the entire pollen sac appears to be of central importance. ABA plays a key role in plant stress responses by regulating the transcription of numerous stress-induced genes. While many species exhibit increased ABA concentrations in floral organs under abiotic stress conditions (such as cold or drought), excessively high ABA levels can impair pollen development and fertility (Saini 1997; Parish et al. 2012). However, maintaining viable pollen alone does not necessarily ensure stable yields. This is exemplified by the SC×SB hybrid, which, despite exhibiting increased pollen production, does not always achieve a proportionally higher seed set (as measured via the PHI). This may represent a short-term compensatory response of the plant that is insufficient to stabilize overall grain formation. The extent to which other factors, such as stigma receptivity or different pollination mechanisms, contribute to this phenomenon remains to be further investigated.

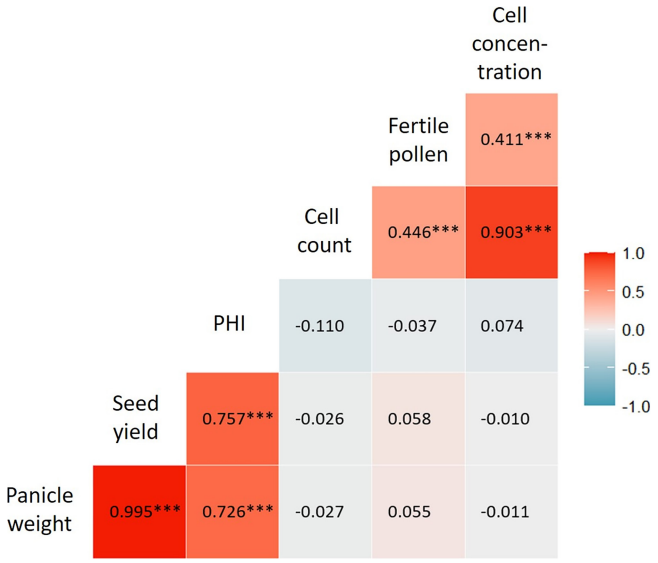

FIGURE 10 | Heatmap illustrating Pearson's correlations among the considered traits: panicle weight (g), seed yield (g), panicle harvest index (PHI), cell count, fertile pollen (%), and cell concentration (cells/mL); strong positive correlations (1) are depicted in red, while strong negative correlations (−1) are represented in blue (significance level: ***0.001; **0.01; *0.05).

Further, no correlation between yield parameters and pollen traits was found, confirming previous results of Schaffasz, Windpassinger, Snowdon, et al. (2019). One reason might be

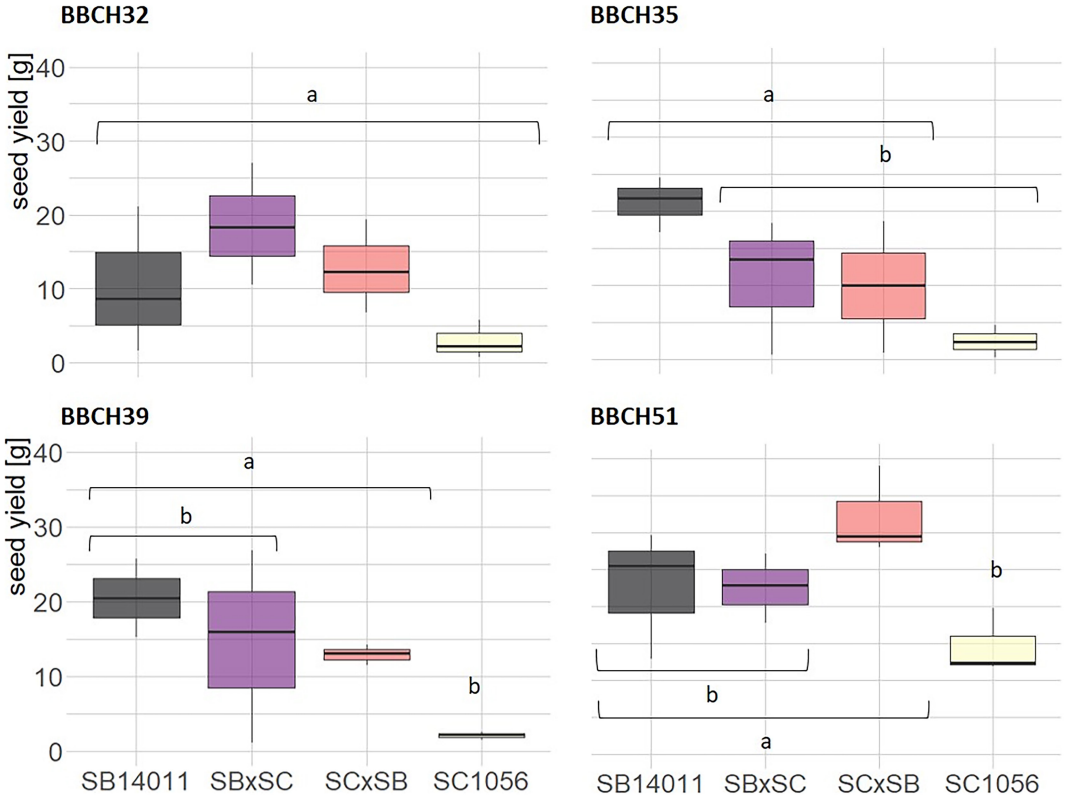

FIGURE 11 | Boxplots showing the seed yield of the cold-tolerant parental line SB14011 and the cold-sensitive parental line SC1056 and their reciprocal F1 hybrids in the different treatment stages (cold stress after BBCH32, BBCH35, BBCH39, and BBCH51); *p*-values in the Supporting Information (Table S1).

TABLE 4 | The result of the *t*-test for the difference between the two forms of pollination (cross-pollination vs. self-pollination) in terms of panicle harvest index (PHI) and seed yield (g).

		PHI	Seed yield
	BBCH	*p*-value	*p*-value
SB14011	BBCH32	0.624	0.844
	BBCH35	0.132	0.0262*
	BBCH39	0.431	0.442
	BBCH51	0.081	0.623
SB×SC	BBCH32	0.661	0.927
	BBCH35	0.559	0.553
	BBCH39	0.518	0.733
	BBCH51	0.818	0.065
SC1056	BBCH32	0.753	0.719
	BBCH35	0.867	0.879
	BBCH39	0.015*	0.093
	BBCH51	0.534	0.912
SC×SB	BBCH32	0.993	0.321
	BBCH35	0.717	0.885
	BBCH39	0.200	0.111
	BBCH51	0.442	0.199

Significance level: ***0.001; **0.01; *0.05.

that, due to the substantial fluctuations in the proportion of fertile pollen, cell concentration, and cell count, it is challenging to establish threshold values that might be necessary for achieving complete seed set. Nevertheless, these findings also suggest pollen traits not being the crucial factor for reduced seed set after cold stress in general, except for sensitive genotypes under certain circumstances. This assumption is reinforced by the lacking effect of cross pollination (see Section 4.3 and Figure 12).

However, it should be noted that the present analysis is based on a limited number of genotypes—two contrasting inbred lines and their reciprocal hybrids. Hence, further studies are required to validate these results on a broader genetic diversity.

4.3 | Restriction of the Female Floral Organ

The receptivity of the pistil has repeatedly been discussed as a factor influencing cold-sensitivity in sorghum (Osuna-Ortega et al. 2003; Thakur et al. 2010). Even in the case of other abiotic stress factors such as drought and heat stress, pollen was initially identified as sensitive to cold, although the receptivity of the pistil later turned out to be equally responsible. Initially, the higher susceptibility of pollen to oxidative stress and the associated damage compared with pistils was discussed (Djanaguiraman et al. 2018). However, recent findings have shown that both pearl millet and sorghum stigmas respond to heat stress to a similar extent and are equally sensitive (Jagadish 2020). Even under drought stress, it could be demonstrated that the male component is not primarily responsible for low yield. Following exposure to water deficiency, although an increase in sterile pollen (up to 9%) was observed, this had no significant impact on the grain formation of the sorghum plants as well (Manjarrez-Sandoval et al. 1989).

Nonetheless, as previously outlined, pollen fertility is still cited as the primary cause of reduced yields in sorghum after cold stress. To further investigate the impact of cold on pollen fertility and pistil receptivity, the genotypes were exposed to cold stress at different reproductive developmental stages and subjected to self-pollination versus cross-pollination by unstressed plants serving as pollen donors.

For the cold-tolerant inbred line SB14011 and the reciprocal F1 hybrids, cross-pollination had no effect on seed yield and PHI across all treatments. Cross-pollination by an unstressed pollen donor only showed an increased PHI in the cold-sensitive genotype SC1056 after cold stress from BBCH39, with no difference in seed yield. However, because the examination of pollen traits did not record a significantly lower proportion of fertile pollen, this effect does not seem likely to be due to the external pollen donor. Instead, partially dead parts of the panicles as a response to cold stress could explain the lower PHI of the self-pollinated plants in this case. In all other treatment stages, i.e., cold stress from BBCH32, BBCH35, and BBCH51 onwards, cross-pollination had no effect on the PHI and seed yield of the cold-sensitive genotype.

Altogether, this study suggests that pollen fertility is not the limiting factor for seed set after cold stress in sorghum. Instead, it appears that the female floral organ is constrained after exposure to cold. This aligns with sorghum's reactions to other abiotic stress factors as described earlier. Furthermore, an increased sensitivity of the female gametophyte to cold stress has been observed in other species, such as *Cryptantha flava*, in the form of irreversible egg cell abortion (Casper 1990), or in chickpeas, in the form of delayed egg cell maturation and increased embryo abortion (Srinivasan et al. 1999). Because the viability of the egg cell is essential for the normal functioning of the pistil (Dumas et al. 1984), it can be assumed that cold stress also affects this aspect in the examined sorghum genotypes.

However, the results of this study are based on a limited number of genotypes—two contrasting inbred lines and their reciprocal hybrids. Generalizing these results for the highly diverse crop sorghum should be done with caution. Multiple, genotype-specific mechanisms may contribute to reproductive cold tolerance, involving both male and female organ responses. The present findings suggest that pollen fertility is not the limiting factor; however, they do not provide conclusive evidence regarding alternative key determinants or physiological mechanisms supporting the involvement of female organs. Stigma receptivity could be a crucial factor that requires further investigation. To validate this hypothesis in future studies, histological analyses, gene expression studies, and hormone analyses could provide valuable insights.

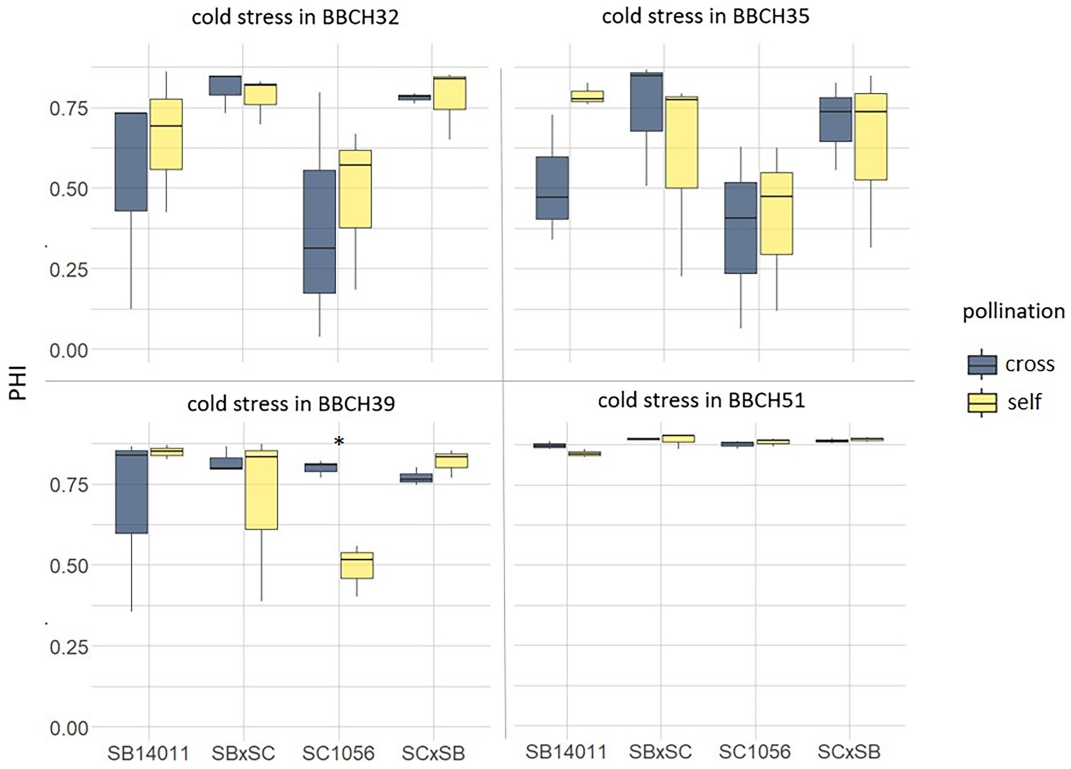

FIGURE 12 | Boxplots showing the panicle harvest index (PHI) after cross-pollination and self-pollination of a cold-tolerant (SB14011) and a cold-sensitive inbred line (SC1056) and their reciprocal hybrids (SB14011 × SC1056 and SC1056 × SB14011) exposed to cold stress at different developmental stages; The significance levels refer to the differences between the pollination forms of a genotype within a treatment stage (significance level: ***0.001; **0.01; *0.05).

4.4 | Evaluation of Pollen and Yield Traits as a Parameter for Assessing Cold Tolerance

In the present study, measurements of pollen fertility, cell concentration, and cell count were conducted using the impedance flow cytometer as described by Heidmann et al. (2016). The PHI and seed yield, as proposed by Krishnamurthy et al. (2014), were used as secondary measures of reproductive cold tolerance. These traits were subsequently compared with each other. The goal of this research was to find an appropriate parameter for assessing and identifying cold-tolerant genotypes to optimize future selection in this area. As expected, the measured yield traits showed strong correlations with each other, just like the measured pollen traits. However, none of the correlations between the yield and pollen traits were statistically significant. This could also be observed in maize plants where it was found that pollen production is important for maximum grain formation, but no direct correlation between pollen production and grain yield was found (Westgate et al. 2003). In addition, this finding is in line with Schaffasz, Windpassinger, Snowdon, et al. (2019). Due to the substantial fluctuations in the proportion of fertile pollen, cell concentration, and cell count, it is challenging to establish threshold values that might be necessary for achieving complete seed set. As more reliable indicators, the PHI or grain weight, which directly reflect seed yield, were identified. Based on the finding that pollen fertility may be of less importance for reproductive cold tolerance than previously assumed, the data collected using the IFC served as complementary parameters that could reveal associations with other potential influencing factors, such as the sensitivity of the female flower organ.

4.5 | Inheritance of Cold Tolerance

In the literature, the complex inheritance of abiotic stress tolerance, including cold tolerance, is frequently emphasized (Thomashow 1999; Sanghera et al. 2011). This complexity underscores the importance of understanding the genetic basis of these traits to enhance the breeding process effectively. To investigate the inheritance of cold tolerance more precisely, reciprocal F1 hybrids were generated through hand emasculation between the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056. By this method, the F1 hybrid has the same cytoplasm as its female parent used to analyze the per se performance of traits. This would not have been the case if we analyzed maintainer × restorer F1 hybrids, using the A1-sterile female line to produce the maintainer × restorer cross (as done in Schaffasz, Windpassinger, Friedt, et al. 2019), since then this F1 hybrid would have another cytoplasm than the fertile maintainer line used to study per se performance for pollen and seed yield traits, implying possible distorting effects.

The analyses showed that the inheritance of cold tolerance in the examined lines depends on the specific developmental stage at which the plants were exposed to cold stress, as well as the duration of exposure. After cold stress at the developmental stages BBCH 35 and BBCH 39, no differences were observed between the parental lines and their hybrids. However, the hybrid SB × SC exhibited a significantly higher yield after cold stress from BBCH 32, and the hybrid SC × SB showed a significantly higher yield after cold stress from BBCH 51 compared with the cold-sensitive mother SC1056. Contrary to the findings of Schaffasz,

Windpassinger, Friedt, et al. (2019), instead of a pronounced heterosis, a mainly additive inheritance can be observed in this hybrid combination. The additive inheritance of QTL contributing to reproductive cold tolerance has already been described in rice (Suh et al. 2010). A statement in favor of the presumed heterotic, dominant inheritance (F1 corresponding to the better parent) can be made in this hybrid combination only for cold stress from BBCH 32 onwards. However, it has to be acknowledged that the present study has only focused on a single hybrid combination, in contrast to 49 F1 hybrid combinations evaluated by Schaffasz, Windpassinger, Friedt, et al. (2019). It is widely accepted that the expression of heterosis is combination-specific and generally tends to increase with a higher genetic distance between the parents. Both inbred lines (SB14011 and SC1056) of the present study originate from the restorer pool of the JLU sorghum breeding program, which may have limited the magnitude of heterosis in the resulting *intra*-pool cross compared with Schaffasz, Windpassinger, Friedt, et al. (2019), where the tested hybrids were *inter*-pool crosses. However, the results regarding the lack of high-parent heterosis are concordant in both studies. Additionally, the present study did not reveal differences between the two reciprocal hybrids, indicating the absence of predominating maternal or paternal inheritance. From a hybrid breeding perspective, there is thus no compelling reason to primarily select for cold tolerance in one pool.

4.6 | Outlook

For a more precise determination of the most critical phase within reproductive development, future studies with a clear distinction between onset and duration of cold stress are necessary. Additionally, phytohormone analyses could be beneficial. They may help to identify the most sensitive stage by providing insights into the phytohormone synthesis of different genotypes following cold exposure. A more detailed understanding of phytohormone responses in cold-sensitive genotypes would enable a better identification of adaptive mechanisms in various developmental stages of cold-tolerant genotypes. Furthermore, the role of stigma receptivity should be further investigated, as it is discussed as a potential key factor for reproductive cold tolerance. This knowledge could facilitate the future identification of potential candidate genes for reproductive cold tolerance in *S. bicolor*.

5 | Conclusion

A deeper understanding of reproductive cold tolerance is necessary for expanding the cultivation of sorghum into temperate regions. This study suggests that pollen fertility is not the principal factor constrained by cold stress as previously assumed, pointing to a higher importance of the female organs. Nevertheless, it should be noted that the analysis included only a limited number of genotypes, which restricts the transferability of the results. Furthermore, the early reproductive phase is confirmed as the most sensitive to cold. Acclimatization after cold stress before BBCH39 in sorghum could not be observed. Additionally, there are no predominating maternal or paternal effects observed in the inheritance of reproductive cold tolerance in F1 hybrids. Future studies should include a greater genetic diversity to further investigate the role of female organs and the genetic determinants of cold tolerance.

Author Contributions

LN planned and supervised climate chamber experiments and data collection, performed the data analysis, interpreted the results, and wrote the manuscript. NK contributed to data analysis. RS received the funding, contributed to devise the study, and edited the manuscript. BW received the funding, contributed to devise the study, and edited the manuscript. SW devised the study, interpreted the results, and edited the manuscript. All authors contributed to the article and approved the submitted version.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All phenotypic data are presented in detail in supplemental file "Data_Neitzert_2024."

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

4 Publikation II

Cold Shock for Cold Tolerance: Phytohormone Dynamics in Sorghum Provides Insights

Luisa Neitzert, Natalja Kravcov, Yudelsy Antonia Tandron Moya, Steffen Windpassinger, Nicolaus von Wirén, Rod Snowdon, Benjamin Wittkop

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RESEARCH ARTICLE OPEN ACCESS

Cold Shock for Cold Tolerance: Phytohormone Dynamics in Sorghum Provides Insights

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Keywords: abscisic acid | climate adaptation | cold sensitivity | gibberellins | jasmonic acid | pollen fertility | reproductive cold tolerance | *sorghum bicolor*

ABSTRACT

The loss of yield due to cold stress during the early reproductive phase poses challenges to the expansion of sorghum cultivation into temperate regions. A better understanding of the physiological mechanisms is crucial for rapid progress in breeding cold-tolerant sorghum varieties. To identify the floral phytohormones responsible for reproductive cold tolerance, a cold-tolerant and a cold-sensitive genotype were subjected to cold stress at various developmental stages during the early reproductive phase. In addition to abscisic acid and its derivatives, including abscisic acid glucose ester, dihydrophaseic acid, and phaseic acid, various gibberellins as well as jasmonic acid and its bioactive form jasmonic acid isoleucine were examined. We found that cold-tolerant sorghum is capable of downregulating abscisic acid concentration under cold stress. While existing literature primarily attributes increased abscisic acid concentration, combined with an insufficient pool of bioactive gibberellins, in sensitive plants as a result of abnormal pollen development, this study shows that this is not the case in sorghum. Additionally, an antagonistic interaction between gibberellins and jasmonic acid was observed regardless of genotype and environmental conditions. These findings contribute to a better understanding of the physiological mechanisms behind cold tolerance in sorghum and could provide important insights for future breeding efforts aiming to accelerate the expansion of cold-tolerant sorghum varieties into temperate climates.

1 | Background

Plants are able to adapt to stressful conditions through changes in physiological processes (Bohnert et al. 1995). A particular focus is on the adaptation of sorghum to cold, which is essential for its expansion into temperate climates. In times of climate change, increasing population pressure, and deteriorating soil quality, sorghum is gaining importance as a robust crop to ensure food and feed supply (Bindraban et al. 2012; Patil 2017). Sorghum presents a promising option for agriculture in temperate regions due to its high adaptability, including the ability to thrive under dry conditions, efficient water usage, and growth on less fertile soils (Berenji and Dahlberg 2004; Zheng et al. 2011; Patil 2017). The added value provided by sorghum cultivation is increasingly sparking interest from both scientists and agronomists to decipher the mechanisms contributing to cold tolerance to expand cultivation in the future. While juvenile cold tolerance is well researched, there is limited knowledge at the physiological level regarding cold tolerance during the reproductive stage. There is a significant need for further research regarding the physiological mechanisms contributing to cold tolerance, particularly phytohormone regulation in sorghum. Abscisic acid (ABA) and gibberellins (GA), both key hormones in cold stress, have already been discovered in rice plants. While cold-tolerant rice plants could maintain low ABA levels through the presence of ABA-regulating genetic sequences (*OSZEP1* and *OSNCED3*), the ABA concentration in cold-sensitive plants was significantly higher (Oliver et al. 2007; Ji et al. 2011; Sharma

and Nayyar 2016). Furthermore, increased degradation of ABA through conjugation or oxidation in cold-tolerant rice plants is thought to reduce ABA levels. Here, the expression of both ABA-8-hydroxylase genes, *ABA8ox1* and *ABA8ox2*, is higher in cold-tolerant genotypes (Nambara and Marion-Poll 2005; Oliver et al. 2007). In addition to ABA regulation, maintaining a sufficiently high pool of bioactive gibberellins to protect the plant from cold damage has been identified, with altered biosynthesis pathways of GA4 and GA7 (Sharma and Nayyar 2016). Besides altered ABA and GA regulation, jasmonic acid is also attributed a key role in involvement in phytohormones contributing to cold tolerance in different poaceae species (Du et al. 2013; Cai et al. 2014). However, problems with hypertrophy of tapetal cells and abnormal pollen growth are described as a result of increased ABA levels and insufficient pools of bioactive gibberellins in the absence of cold tolerance. Suppression of the cell wall-bound acid invertase gene and the monosaccharide transporter gene disrupts sugar transport to the tapetum, resulting in abnormal pollen development and reduced grain number (Oliver et al. 2005; Mamun et al. 2006). In contrast, reduced fertility of the female floral organ has been discussed as a possible cause for the reduced grain set in cold-sensitive genotypes (Osuna-Ortega et al. 2003, Neitzert et al. 2025).

This work aims to gain a deeper understanding of the physiological mechanisms related to phytohormones in reproductive cold tolerance in sorghum by addressing several open questions: (I) Which phytohormones are down- or up-regulated under cold stress? (II) What differences exist in phytohormone response between cold-tolerant and cold-sensitive genotypes? (III) How do phytohormone levels differ between developmental stages during the early reproductive phase? (IV) To what extent can these results be transferred

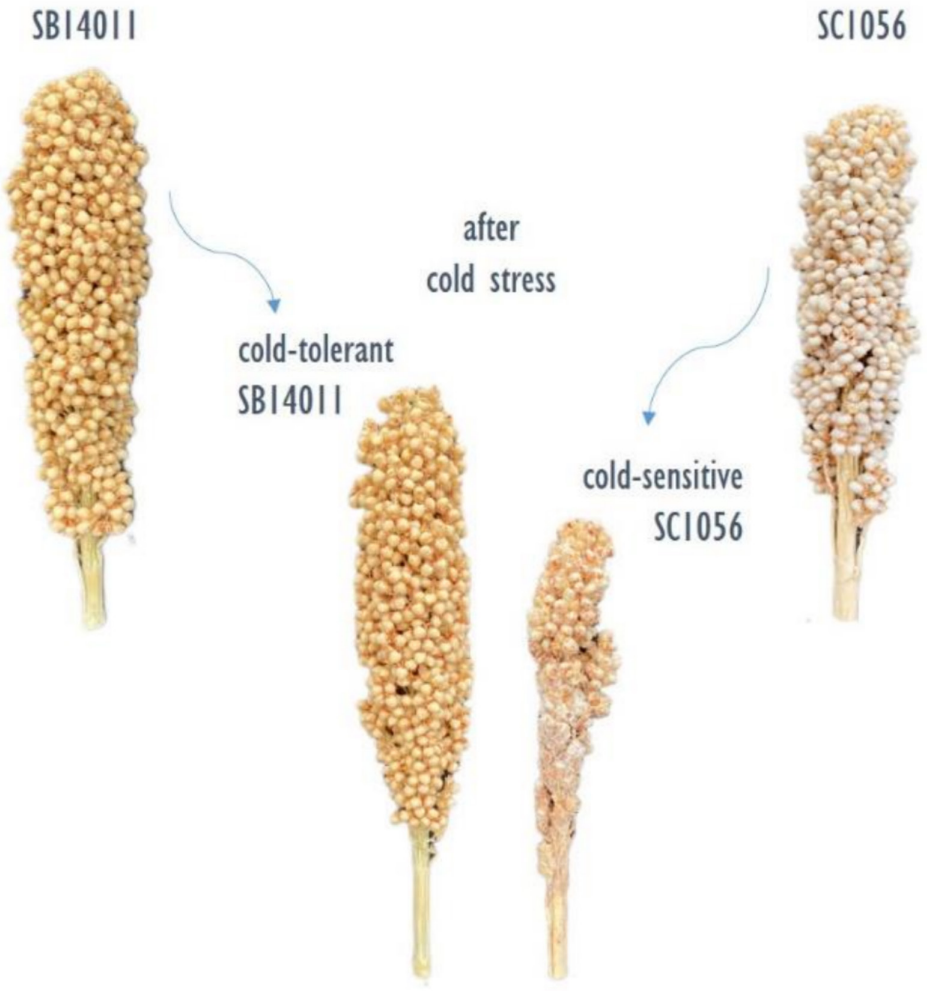

FIGURE 1 | cold-tolerant parental line SB14011 and cold-sensitive parental line SC1056 grown under optimal and stress conditions (see Table 1).

TABLE 1 | Settings of the climate chamber experiment.

	Temperature in Celsius	Day/night cycle in hours	Rel. air humidity
Optimum conditions	30/24	13/11	60%
Cold stress	25/7	13/11	60%

from existing literature on related species? Overall, these findings will contribute to a better understanding of the physiological mechanisms behind cold tolerance in sorghum and could provide important insights for future breeding of cold-tolerant sorghum varieties to accelerate their expansion into temperate climates.

2 | Material and Methods

2.1 | Plant Material

For the experiments, one cold-tolerant (SB14011) and one cold-sensitive (SC1056) inbred line were studied (see Figure 1). The two inbred lines were selected from a diversity set of *Sorghum bicolor* ($n = 330$) consisting of inbred lines of different origin, utilization and subspecies. Traits such as seed yield and panicle harvest index (PHI) under cold conditions were used to evaluate reproductive cold tolerance (Chakrabarty et al. 2021). Thus, genotype SB14011, a breeding line developed and selected under german conditions, was identified as cold-tolerant and SC1056, a conversion line originating from Sudan (Stephens et al. 1967), as cold-sensitive.

2.2 | Defining BBCH-Stages

In this study, we investigated the effects of cold exposure on plants at specific developmental stages. The BBCH scale was used for the standardized coding of these stages. This scale is based on the cereal scale developed by Zadoks et al. (1974) and subdivides plant development into macro- and microstages. The currently applied extended BBCH scale was jointly developed by the Federal Biological Research Centre for Agriculture and Forestry (BBA), the Federal Plant Variety Office (BSA), and the Agricultural Industry Association (IVA). The acronym BBCH is derived from these german institutions: **B**iologische Bundesanstalt für Land- und Forstwirtschaft, **B**undessortenamt, and **Ch**emische Industrie. In this study, the effects of cold were analyzed starting from BBCH35, BBCH39, and BBCH51 (see Figure 2). At BBCH35, the plants had reached the 5-node stage, indicating that the 5th node was at least 2 cm away from the 4th node. During this stage, the flag leaf was notably small, positioned relatively low, and tightly curled up. At BBCH39, the flag leaf had fully developed, exhibiting visible ligules. Finally, at BBCH51, the heading process had initiated, characterized by the emergence of the panicle tip from the leaf sheath (according to the Technology and Promotion Centre (TFZ), BBCH code for sorghum).

2.3 | Climate Chamber Experiments

The climate chamber experiments were conducted at the IFZ Research Center for Biosystems at Justus Liebig University in Giessen, Germany following the procedure described by Schaffasz et al. (2019) with minor modifications. The genotypes were tested under controlled cold stress and optimal growth conditions at different developmental stages (BBCH35, BBCH39, and BBCH51) in the reproductive stage. The plants were grown in 17 × 17 × 17 cm pots filled with Fruhstorfer type N soil and were irrigated and fertilized following good horticultural practices to exclude variation in conditions other than thermal stress. All plants were cultivated under optimal conditions (30°C/13 h during the day and 24°C/11 h at night, with a relative humidity of 60% and LED lighting) during emergence and early vegetative growth. For each of the mentioned BBCH stages, randomly selected plants were transferred to a cold stress chamber (25°C/13 h during the day and 7°C/11 h at night, with a relative humidity of 60% and LED lighting) and exposed to cold stress for 3 days. Three plants were stressed for each genotype and BBCH stage. Under optimal conditions, the panicles were harvested 1 day after reaching the respective BBCH stage. The individual panicles were considered biological replicates, with two technical replicates each for statistical analysis.

2.4 | Phytohormone Analysis

The phytohormones analyzed were abscisic acid (ABA), abscisic acid glucose ester (ABAGe), dihydrophaseic acid (DHPA), phaseic acid (PA), jasmonic acid (JA), jasmonic acid isoleucine (JAIle), gibberellins (GA). Gibberellins GA1, 3, 4, 5, 6, 7, 8, 9, 20, 29, 51 were included in the analysis but were not detected in the samples. GA 12, 15, 19, 24, 44, 53 were detected. Organic solvents, methanol (MeOH) and acetonitrile (ACN), were purchased from Th. Geyer GmbH & Co. KG, Germany. Formic acid

FIGURE 2 | Overview of the developmental stages to be examined—BBCH35: flag leaf not yet visible, shortly before the appearance of the leaf tip; BBCH39: ligule of the flag leaf visible; BBCH51: emergence of the panicle.

(FA) was obtained from Biosolve Chimie, France. De-ionized distilled water from the Milli-Q Reference System, Merck, Germany, was used for preparation of all mixtures and as mobile phase.

Standards ABA, ABAGe, JA, JAIle, and GA as well as deuterium labeled compounds used as internal standards were purchased from OlChemim s.r.o, Czech Republic. Standards DHPA and PA were purchased from the National Research Council of Canada, Saskatoon, Canada.

Plant material was ground in liquid nitrogen. Samples of ca. 30 mg (fresh weight) were weighed into 2 mL safe lock tubes (Eppendorf AG, Germany) and kept at −80°C until extraction. Empty tubes were used as blanks. Before extraction, two 3 mm ceria-stabilized zirconium oxide beads were placed into each tube. The samples were extracted and purified as described in Šimura et al. (2018) with some modifications. Briefly, 1 mL ice-cold 50% aqueous (v/v) ACN containing the internal standards was added to each tube for phytohormone extraction. Samples were homogenized in a MM 301 vibration mill (Retsch GmbH, Germany) operating at a frequency of 27 Hz for 5 min and afterward sonicated for 3 min at 4°C using a Sonorex ultrasonic bath (BANDELIN electronic GmbH, Germany). Samples were subsequently extracted using a Reax 32 overhead shaker (Heidolph Instruments GmbH, Germany) for at least 30 min. After 10 min centrifugation at 14000 rpm and 4°C (CT 15 RE centrifuge, Himac, Japan), the supernatant was transferred to clean Eppendorf tubes. All samples were purified using Oasis PRIME HLB RP (1 cc per 30 mg), polymer-based SPE cartridges (Waters Co., USA). After loading the supernatant, the flow-through fraction was collected in a clean tube. The cartridge was then rinsed with 1 mL of 30% (v/v) ACN, and this fraction was collected in the same tube as the flow-through fraction. After this single-step SPE, the samples were evaporated to dryness at 40°C in a vacuum concentrator RVC 2–33 IR (Martin Christ GmbH, Germany) and stored at −20°C until analysis. For analysis, the samples were dissolved in 50 µL of 30% ACN (v/v) containing 0.1% FA and transferred to insert-equipped vials. The analysis of targeted phytohormones was performed by Ultra High Pressure Liquid Chromatography-Heated Electrospray Ionization-High Resolution Mass Spectrometry (UHPLC-HESI-HRMS) technique.

Compound baseline separation was achieved on a reversed phase Acquity UPLC HSS T3 column (10 Å, 2.1 × 100 mm, 1.8 µm, Waters) using a simple gradient elution of A (Water, 0.1% FA) and B (ACN, 0.1% FA) as follows: 0–5 min, 10% B; 5–10 min, 10% to 80% B. Five additional minutes were added for column washing and re-equilibration. In the case of GA, the gradient elution consisted of A (Water, 0.1% FA) and B (MeOH, 0.1% FA) as follows: 0–0.3 min, 10% B; 0.3–0.7 min, 10% to 30% B; 0,7–2 min, 30% to 50% B; 2–4 min, 50% to 60% B; 4–8 min, 60% to 80% B; 8–9.5 min, 80% to 99% B; 9.5–10,4 min, 99% B. The column temperature was set at 45°C and the flow rate at 0.5 mL/min (0.3 mL/min for GA). To preserve the integrity of the column a guard column (130 Å, 2.1 × 5 mm, 1.8 µm, Waters) was used. The injection volume was 5 µL. The UHPLC system (Vanquish Horizon, Thermofisher, San Jose, CA, USA) was coupled to a Q Exactive Plus Mass Spectrometer (Thermofisher, San Jose, CA, USA) equipped with a HESI source operating in negative ion mode. Source values were set as follows: Spray voltage 2.5 kV; Capillary temperature 255°C; S-lens RF level 40; Aux gas heater temp 320°C; Sheath gas flow rate 47; Aux gas flow rate 11. For spectra acquisition a FullMS/dd-MS^2 experiment was performed. Resolution in Full Scan was set as 70,000. For MS/MS experiments resolution 17,500 and NCE 40 V were used. MS data were acquired and processed by Trace Finder Software (v. 4.1, Thermo Scientific, San Jose, CA, USA). Twelve-point curve was prepared from standards mix solutions in the range of 0.5 to 1000 nM. To generate the calibration curve, the peak area on the extracted ion chromatogram (XIC) of the deprotonated molecule ion [M-H]- was measured. A least-square linear regression was used to best fit the linearity curve.

2.5 | Statistical Analysis

A Welch two-sample *t*-test was conducted to analyze significant differences in the investigated phytohormone concentrations between stress treatment and optimal conditions at specific treatment stages (BBCH35, BBCH39, BBCH51) separately for each genotype.

To test for differences between the two genotypes, a paired *t*-test was performed to compare each individual treatment stage (BBCH35, BBCH39, and BBCH51). This was done separately for the two conditions (cold stress and optimal conditions).

Separately, a one-way ANOVA analysis was conducted for each trait to assess possible differences between treatment stages (BBCH35, BBCH39, BBCH51) within each genotype. This was also done separately for the two conditions (cold stress and optimal conditions). The independent variable in each analysis was "treatment", and the dependent variable was the respective trait. These traits included the phytohormones abscisic acid, abscisic acid glucose ester, dihydrophaseic acid, phaseic acid, jasmonic acid, jasmonic acid isoleucine, gibberellins (gibberellic acid 12, 15, 19, 24, 44, and 53).

After each ANOVA, a Least Significant Difference (LSD) post hoc analysis was performed to determine which treatments showed statistically significant differences from each other.

The Pearson correlation coefficients were used to evaluate the relationships between phytohormones, pollen traits, and yield parameters. Separate correlations were performed for the two genotypes (SB14011 and SC1056) and the two treatments (control and stress).

The statistical analysis and presentation of results were conducted in R version 4.0.5.

3 | Results

3.1 | Impact of Cold Stress on Phytohormone Levels

Significant differences in various phytohormone concentrations were seen at all developmental stages for both the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056 during the control and stress treatments (Table 2).

TABLE 2 | Results of the Welch two-sample *t*-test analyzing significant differences between control and stress conditions across various treatments (BBCH35, BBCH39, and BBCH51), separately for the genotypes SB14011 and SC1056, concerning different phytohormones, including abscisic acid (ABA), abscisic acid glucose ester (ABAGe), dihydrophaseic acid (DHPA), jasmonic acid (JA), phaseic acid (PA), gibberellins (GA), and its derivatives GA12, GA15, GA19, GA24, GA44, and GA53; (Significance level: *** 0.001; ** 0.01.; * 0.05).

Genotype	**SB14011**			**SC1056**		
Contrasts	**BBCH35**	**BBCH39**	**BBCH51**	**BBCH35**	**BBCH39**	**BBCH51**
Phytohormone			*p*			
ABA	0.1321	0.5938	0.067	0.0105*	8.57E-05***	0.0064**
ABAGe	0.0574	0.1182	0.3632	0.2979	NA	0.009**
DHPA	0.6897	0.6602	0.0072**	0.3439	0.0584	0.8204
PA	0.0127*	0.0707	0.005**	0.0198*	0.0026**	0.8534
GA	0.8298	0.0022**	0.1826	0.16	0.0003***	0.02033*
JA	0.4113	0.6635	0.1615	0.2911	0.1098	0.0725
JAIle	0.42	0.1368	0.8257	0.2721	0.235	0.1645
GA12	0.1323	NA	NA	0.1531	NA	NA
GA15	0.0685	NA	NA	NA	NA	NA
GA19	0.5613	0.0016**	0.7392	0.101	9.02E-05***	0.6145
GA24	0.0315*	NA	NA	0.042*	NA	0.3632
GA44	0.9153	0.0005***	0.2637	0.2264	0.0009***	0.1106
GA53	0.498	0.0349*	0.0346*	0.1412	0.0034**	0.8208

For genotype SB14011, at BBCH35 under stress conditions compared to the control, significant differences were observed exclusively in the concentration of phaseic acid (PA). In BBCH39, significant differences were observed in the concentrations of the gibberellin (GA) derivatives GA19, GA44, and GA53 depending on the conditions. Similarly, significant differences in PA concentration were observed at BBCH51, along with significant disparities in the concentrations of dihydrophaseic acid (DHPA) and GA53.

In the case of genotype SC1056, significant differences between control and stress conditions were observed for the hormones abscisic acid (ABA), PA, and the gibberellin derivatives GA24 at BBCH35. At BBCH39, significant differences in ABA, PA, GA, and the gibberellin derivatives GA19, GA44, and GA53 were observed depending on the treatment. At BBCH51, a significant difference in ABA concentration between stress and control conditions was also noted, along with significant differences in the concentrations of abscisic acid glucose ester (ABAGe) and GA.

The differences in phytohormone concentrations for the cold-tolerant genotype SB14011 under stress and control conditions at various developmental stages (BBCH35, BBCH39, and BBCH51) are illustrated in Figure 3. The bar graphs highlight significant differences in phytohormone concentrations, including dihydrophaseic acid (DHPA), phaseic acid (PA), gibberellins (GA), and their derivatives between the two conditions.

At BBCH35, SB14011 exhibits a significantly higher PA concentration under stress conditions compared to the control. Conversely, at BBCH39, the GA concentration and the concentrations of its derivatives GA19, GA44, and GA53 are significantly lower under stress conditions. Additionally, at BBCH51, alongside the elevated PA and GA53 concentrations, a reduced DHPA concentration is observed compared to the control.

Significant differences in the concentrations of abscisic acid (ABA), abscisic acid glucose ester (ABAGe), phaseic acid (PA), gibberelins (GA), and their derivatives are apparent when considering the phytohormone concentrations of the cold-sensitive genotype in Figure 4 under stress versus control conditions across various developmental stages (BBCH35, BBCH39, and BBCH51). Particularly, the significantly higher ABA concentrations under stress conditions observed across all developmental stages are notable. Furthermore, at BBCH35, PA and GA24 concentrations are also significantly elevated under stress conditions. In addition to the increased ABA and PA concentrations, BBCH39 exhibits significantly lower GA concentrations under stress conditions. Furthermore, at BBCH39, the concentrations of GA derivatives GA19, GA44, and GA53 are significantly reduced. Finally, at BBCH51, ABAGe concentrations are decreased, while GA concentration are increased under stress conditions.

Figure 4 illustrates the dynamic changes in phytohormone concentrations depending on environmental conditions and genotypes during plant development.

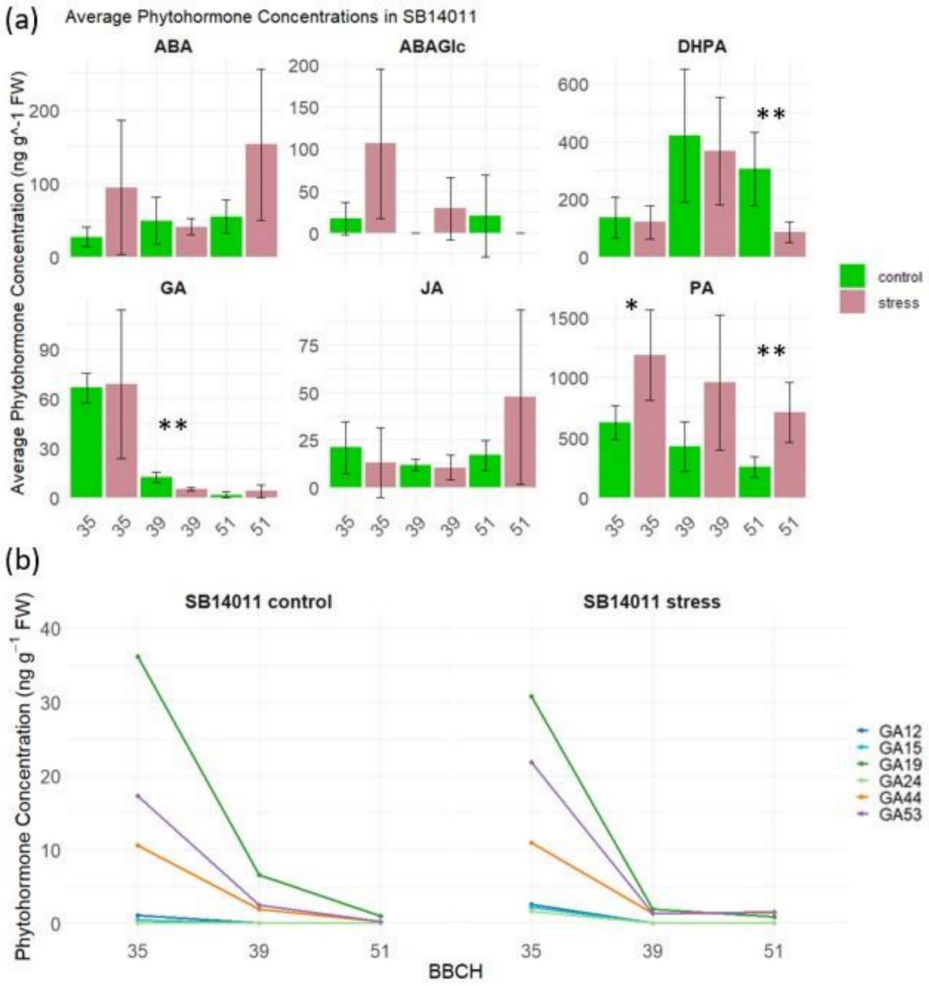

FIGURE 3 | (a) Comparison of phytohormone concentrations including Abscisic Acid (ABA), Abscisic Acid Glucose Ester (ABAGe), Dihydrophaseic Acid (DHPA), Gibberellins (GA), Jasmonic acid (JA), and Phaseic Acid (PA) between stress and control conditions of genotype SB14011 at different developmental stages (BBCH35, BBCH39, and BBCH51); (b) Comparison of phytohormone concentrations of Gibberellin derivatives including GA12, GA15, GA19, GA24, GA44, and GA53 between stress and control conditions of genotype SB14011 at different developmental stages (BBCH35, BBCH39, and BBCH51).

3.2 | Comparison of Phytohormone Levels Between Genotypes Under Cold Stress

The comparison of phytohormone levels between the cold-tolerant line SB14011 and the cold-sensitive line SC1056 reveals significant differences in all developmental stages under both control and stress conditions (Table 3).

Under control conditions, the genotypes differed at BBCH35 in abscisic acid (ABA) and the gibberellins (GA) GA15, GA19, GA44, and GA53: SC1056 showed higher ABA but lower GA concentrations. At BBCH39, only GA44 differed, and at BBCH51, jasmonic acid (JA) was higher in SC1056.

Under stress conditions, significant differences in ABA were detected at all stages, with consistently higher levels in the cold-sensitive genotype SC1056. Additionally, at BBCH35, the GA15 concentration is significantly lower in SC1056. Furthermore, at BBCH51, the ABAGe concentration is significantly higher, while the PA concentration is significantly lower in the cold-sensitive genotype.

Figure 5 illustrates these trends: ABA levels in the cold-sensitive genotype SC1056 are markedly higher than those in the cold-tolerant genotype SB14011 at all developmental stages. While the ABA concentration of SC1056 under stress conditions averaged 1019.3 at BBCH35, 66.45 at BBCH39, and 424.8 at BBCH51, SB14011 exhibited values of only 94.41, 41.11, and 152.86, respectively. This trend is visually represented in Figure 3. Considering all phytohormones collectively, it is noticeable that the phytohormone level after cold stress in BBCH35 of the cold-sensitive genotype, exceeding 2000, is significantly higher than that of the cold-tolerant genotype. Additionally, for both genotypes, the overall phytohormone level is higher under stress conditions compared to control conditions.

Figure 6 depicts the phytohormone levels of individual gibberellin derivatives in the two genotypes SB14011 and SC1056 under stress and control conditions. It can be observed that under stress conditions, the concentration of GA15 at BBCH35 is detectable only in the cold-tolerant genotype SB14011 (2.227 ng), while it is absent in SC1056.

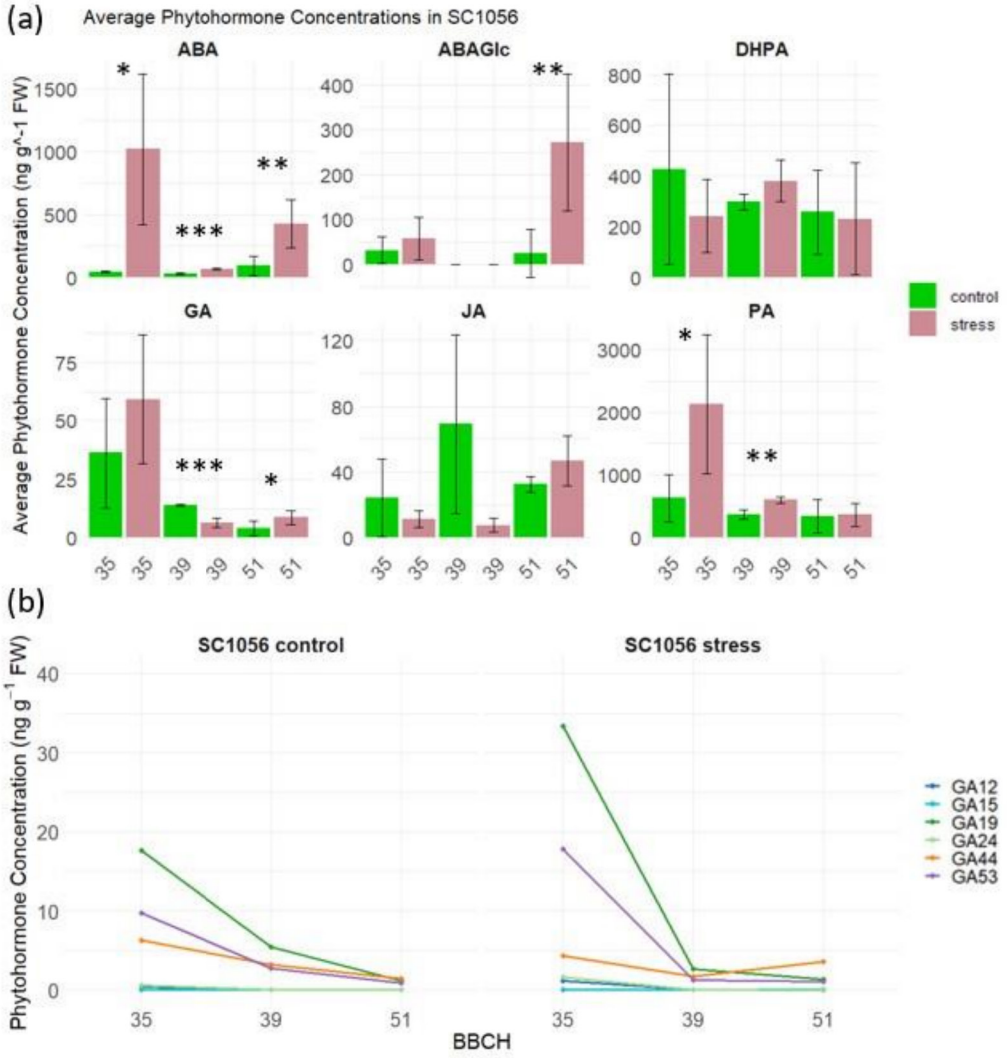

FIGURE 4 | (a) Comparison of phytohormone concentrations including abscisic acid (ABA), abscisic acid glucose ester (ABAGe), dihydrophaseic acid (DHPA), gibberellins (GA), jasmonic acid (JA), and phaseic acid (PA) between stress and control conditions of genotype SC1056 at different developmental stages (BBCH35, BBCH39, and BBCH51). (b) Comparison of phytohormone concentrations of Gibberellin derivatives including GA12, GA15, GA19, GA24, GA44, and GA53 between stress and control conditions of genotype SC1056 at different developmental stages (BBCH35, BBCH39, and BBCH51).

3.3 | Differences in Phytohormone Levels Between Developmental Stages

3.3.1 | Abscisic Acid and Abscisic Acid Glucose Ester

No significant differences in abscisic acid (ABA) and abscisic acid glucose ester (ABAGe) levels were observed among the investigated developmental stages for both the cold-tolerant and the cold-sensitive genotypes under control conditions (Table 4). While in SB14011, the ABA concentration did not differ among developmental stages even under stress conditions, SC1056 exhibited significant differences between developmental stages BBCH35 and BBCH39, as well as between BBCH35 and BBCH51.

For ABAGe, both genotypes showed stage-dependent differences: SB14011 between BBCH35 and BBCH39 and BBCH35 and BBCH51, and SC1056 between BBCH35 and BBCH51 and BBCH39 and BBCH51.

When examining the ABA concentrations of the cold-sensitive genotype SC1056 in Figure 7a under stress conditions at different developmental stages (BBCH35, BBCH39, and BBCH51), significant differences become apparent. Particularly, the significantly higher ABA concentrations following cold stress from BBCH35 onwards are mentionable. Under control conditions and for the cold-tolerant genotype SB14011, even under stress conditions, ABA concentrations consistently remain below 100 ng g^{-1} FW. In Figure 7b, the significantly higher ABAGe concentration following cold stress in SC1056 at BBCH51 and in SB14011 at BBCH35 compared to other treatments within each genotype is clearly evident.

3.3.2 | Dihydrophaseic Acid and Phaseic Acid

When comparing the phytohormone levels of dihydrophaseic acid (DHPA), significant differences among developmental stages were observed only under control conditions in the

TABLE 3 | The results of the *t*-test for the difference between the two genotypes (SB14011 vs. SC1056) in terms of phytohormone concentrations including Abscisic Acid (ABA), Abscisic Acid Glucose Ester (ABAGe), Dihydrophaseic Acid (DHPA), Jasmonic acid (JA), and Phaseic Acid (PA), Gibberellins (GA), Gibberellin derivatives including GA12, GA15, GA19, GA24, GA44, and GA53 between stress and control conditions of genotype SC1056 at different developmental stages (BBCH35, BBCH39, and BBCH51); (significance level: *** 0.001; ** 0.01.; * 0.05).

Contrasts	**SB14011—SC1056**								
Condition	**Control**								
Treatment	**BBCH35**			**BBCH39**			**BBCH51**		
Phytohormones	**df**	***t*-value**	***p***	**df**	***t*-value**	***p***	**df**	***t*-value**	***p***
ABA	7.8474	−2.874	0.02112*	5.6387	1.4504	0.2002	4.5912	−1.0998	0.3257
ABAGe	6.6879	−0.8885	0.4051	/	/	/	8.3091	−0.12456	0.9038
DHPA	4.2334	−1.7092	0.1586	5.2766	1.2922	0.25	7.4214	0.51243	0.6232
PA	4.941	−0.026435	0.9799	6.7169	0.69274	0.5117	4.7095	−0.66461	0.5374
GA	5.0351	2.819	0.03686*	5.3369	−0.34405	0.744	5.2648	−1.212	0.2771
JA	6.2367	−0.27242	0.7941	3.0115	−2.0955	0.1268	8.221	−4.0755	0.003358**
JAIle	7.181	−0.25419	0.8065	3.0015	−1.7144	0.1849	7.8851	−0.47882	0.6451
GA12	4.6332	2.3627	0.0686	/	/	/	/	/	/
GA15	5	3.1399	0.02567*	/	/	/	/	/	/
GA19	4.9132	3.0798	0.02812*	6.0219	1.3928	0.2129	7.8151	−0.64674	0.5363
GA24	4	−2.1205	0.1013	/	/	/	/	/	/
GA44	6.1402	3.1056	0.02034*	6.4072	−13.306	6.61E-06***	4.7336	−1.2661	0.2642
GA53	5.1883	2.4492	0.05621	5.6695	−0.77985	0.4668	5.2098	−1.1579	0.2972
Condition	**Stress**								
Treatment	**BBCH35**			**BBCH39**			**BBCH51**		
Pyhtohormones	**df**	***t*-value**	***p***	**df**	***t*-value**	***p***	**df**	***t*-value**	***p***
ABA	5.2319	−3.7407	0.01235*	8.2188	−4.9401	0.001048**	7.6644	−3.0678	0.01622*
ABAGe	7.5898	1.2013	0.2658	5	1.8845	0.1182	5	−4.3387	0.007438**
DHPA	6.5401	−1.8984	0.1024	6.8206	−0.17586	0.8655	5.2641	−1.5812	0.1717
PA	6.1319	−1.9629	0.09627	5.0985	1.5528	0.1801	9.1613	2.7674	0.02149*
GA	8.2458	0.5183	0.6179	5.733	−1.2977	0.2441	9.2204	−1.2173	0.2537
JA	5.7643	0.19537	0.8518	8.4712	0.86357	0.4116	6.1043	0.051116	0.9609
JAIle	5.8347	0.37994	0.7174	8.5983	−0.68696	0.5102	9.9298	1.3135	0.2186
GA12	7.025	1.5605	0.1625	/	/	/	/	/	/
GA15	5	2.8652	0.03519*	/	/	/	/	/	/
GA19	9.3712	−0.24661	0.8105	8.9368	−2.2459	0.05155	9.7768	−1.7846	0.1053
GA24	0.077026	6.8615	0.9408	/	/	/	5	−1	0.3632
GA44	5.6961	2.3912	0.05615	5.6619	−1.6602	0.1509	9.9307	−1.7041	0.1194

cold-tolerant genotype SB14011 (Table 5), specifically between BBCH35 and BBCH39. No significant differences were observed in the cold-sensitive genotype SC1056 under control conditions. Under stress, DHPA levels remained constant across stages in both genotypes.

Regarding the phaseic acid (PA) levels, SB14011 exhibited no significant differences between developmental stages under either control and stress conditions. SC1056 only showed significant differences under stress conditions between BBCH35 and BBCH39, as well as BBCH35 and BBCH51.

When considering the DHPA and PA concentrations in the cold-sensitive genotype SC1056 (Figure 8), no fluctuations between developmental stages are observed under optimal conditions. However, in the cold-tolerant genotype SB14011,

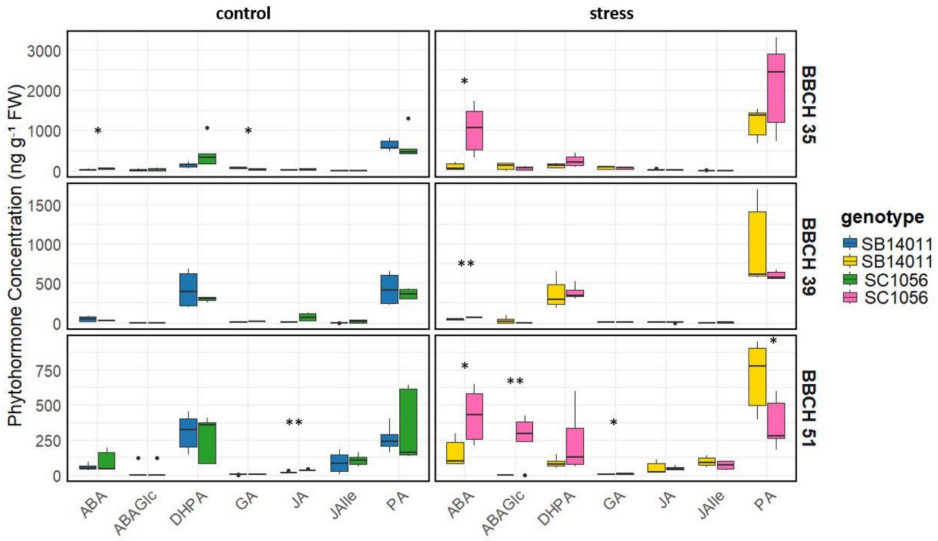

FIGURE 5 | Comparison of phytohormone levels including abscisic acid (ABA), abscisic acid glucose ester (ABAGe), dihydrophaseic acid (DHPA), gibberellins (GA), jasmonic acid (JA), and phaseic acid (PA) between the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056 at different developmental stages (BBCH35, BBCH39, and BBCH51) for both stress and control conditions separately.

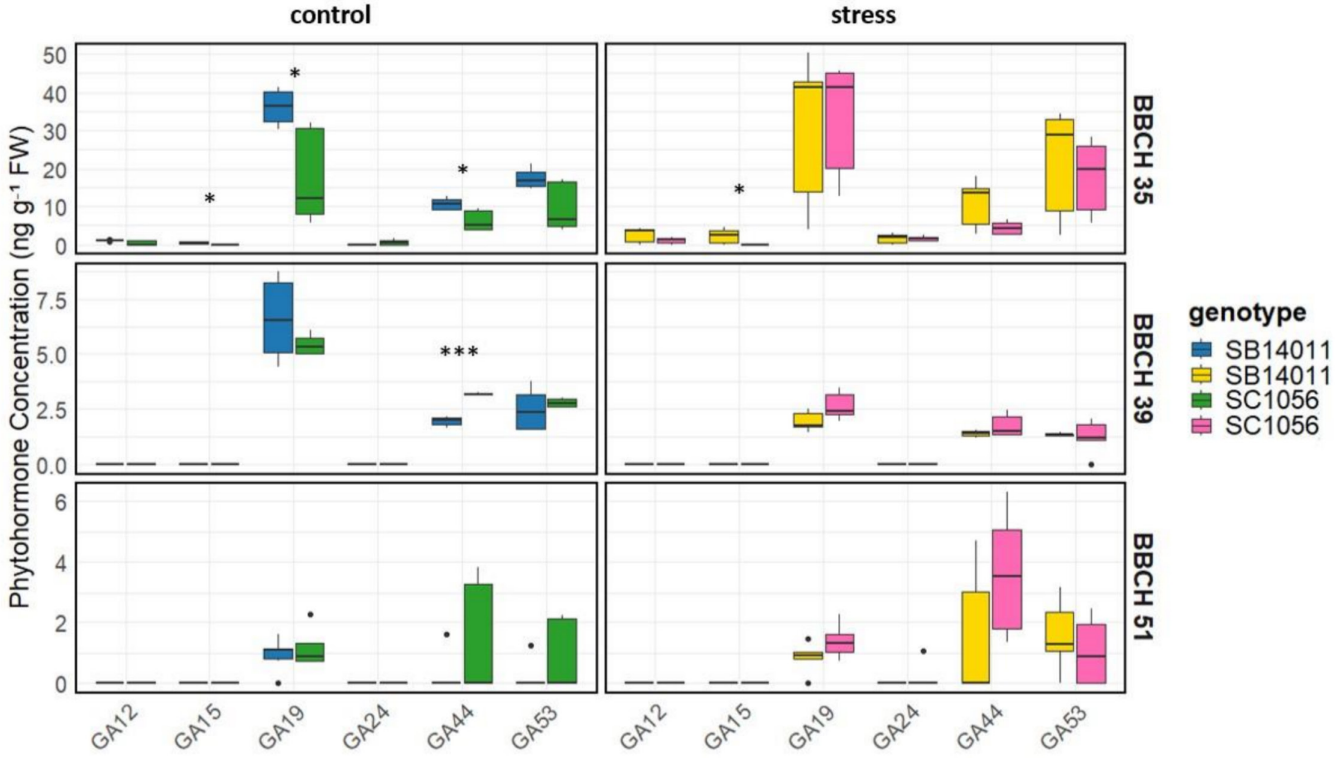

FIGURE 6 | Comparison of phytohormone levels of gibberellin derivatives including GA12, GA15, GA19, GA24, GA44, and GA53 between the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056 at different developmental stages (BBCH35, BBCH39, and BBCH51), separately for stress and control conditions of.

Figure 6 clearly illustrates that DHPA accumulation is significantly lower in BBCH35 compared to other developmental stages (BBCH39 and BBCH51). Regarding PA, no variations between stages can be foundin SB14011. However, in both genotypes, a continuous decline in PA concentration from BBCH35 to BBCH51 is observed. Particularly noteworthy is the significantly increased concentration of almost 2500 ng g^{-1} FW of the SC1056 genotype after cold stress at BBCH35, which is remarkably higher than that of the other two treatment levels, BBCH39 and BBCH51.

3.3.3 | Jasmonic Acid and JA-Isoleucine

When comparing the phytohormone levels of jasmonic acid (JA), significant differences between developmental stages were

TABLE 4 | Results of the ANOVA and subsequent Tukey test examining the phytohormone concentration of abscisic acid (ABA) and abscisic acid glucose ester (ABAGe) at various developmental stages under control and stress conditions for the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056 (significance level: *** 0.001; ** 0.01.; *0.05).

		ABA					ABAGe				
SB14011	**ANOVA**	***p* = 0.143**					***p* = 0.491**				
Condition	**Contrasts**	**Estimate**	**SE**	**df**	**t.ratio**	***p***	**Estimate**	**SE**	**df**	**t.ratio**	***p***
	BBCH35-BBCH39	/	/	/	/	/	/	/	/	/	/
Control	BBCH35-BBCH51	/	/	/	/	/	/	/	/	/	/
	BBCH39-BBCH51	/	/	/	/	/	/	/	/	/	/
SC1056	ANOVA	*p* = 0.133					*p* = 0.463				
	BBCH35-BBCH39	/	/	/	/	/	/	/	/	/	/
Control	BBCH35-BBCH51	/	/	/	/	/	/	/	/	/	/
	BBCH39-BBCH51	/	/	/	/	/	/	/	/	/	/
SB14011	ANOVA	*p* = 0.0824					*p* = 0.0135 *				
	BBCH35-BBCH39	/	/	/	/	/	77.6	32.2	15	2.406	0.0295
Stress	BBCH35-BBCH51	/	/	/	/	/	106.3	32.2	15	3.297	0.0049
	BBCH39-BBCH51	/	/	/	/	/	28.7	32.2	15	0.891	0.3872
SC1056	ANOVA	*p* = 0.00138 **					*p* = 0.000328 ***				
	BBCH35-BBCH39	953	210	15	4.548	0.0004	56.8	53.5	15	1.061	0.3055
Stress	BBCH35-BBCH51	594	210	15	2.837	0.0125	−215.2	53.5	15	−4.019	0.0011
	BBCH39-BBCH51	358	210	15	−1.71	0.1078	−272	53.5	15	−5.08	0.0001

observed only under stress conditions for the cold-sensitive genotype SC1056 (see Table 6). Here, significant differences were noted between developmental stages BBCH35 and BBCH51, as well as BBCH39 and BBCH51.

In contrast, developmental stages showed significant differences in terms of jasmonic acid-isoleucine (JA-Ile) for both genotypes under both control and stress conditions. Both SB14011 and SC1056 exhibited significant differences between developmental stages BBCH35 and BBCH39, as well as between BBCH35 and BBCH51 in both control and stress conditions.

In Figure 9a, the cold-sensitive genotype SC1056 shows a pronounced increase in JA concentration after cold stress at BBCH51, whereas the increase observed under optimal conditions at BBCH39 is not significant compared to the other stages. Figure 9b shows that JA-Ile concentrations steadily increase from BBCH35 to BBCH51 in both genotypes, regardless of conditions, with the highest accumulation at BBCH51. This stage differs significantly from BBCH35 and BBCH39, which show similar concentrations.

3.3.4 | Gibberellins

Examination of the phytohormone levels of gibberellins (GA12, GA15, GA19, GA24, GA44, and GA53) revealed no significant differences between developmental stages under control conditions for the cold-tolerant genotype. However, under stress conditions, significant differences were observed between developmental stages BBCH35 and BBCH39, as well as BBCH35 and BBCH51 (Table 7).

The cold-sensitive genotype SC1056 exhibited significant differences between developmental stages under both control and stress conditions. In both cases, BBCH35 differed from BBCH39 and BBCH51.

It is evident that in both genotypes, regardless of the condition, GA concentration consistently decreases from the early developmental stage BBCH35 through BBCH39 to the later BBCH51 (Figure 10). The highest accumulation is observed in BBCH35 for the cold-tolerant genotype under stress conditions. Interestingly, the GA concentration of the cold-sensitive genotype under stress conditions is comparable to that of the cold-tolerant genotype under optimal conditions.

3.4 | Correlation Analysis of Phytohormones, Pollen Traits, and Yield Parameters

The Pearson correlation was calculated to examine possible relationships between phytohormones, pollen traits, and yield parameters. The yield and pollen data were taken from the dataset of Neitzert et al. (2025). The results for both genotypes under stress conditions are shown in Figure 11 and Figure 12

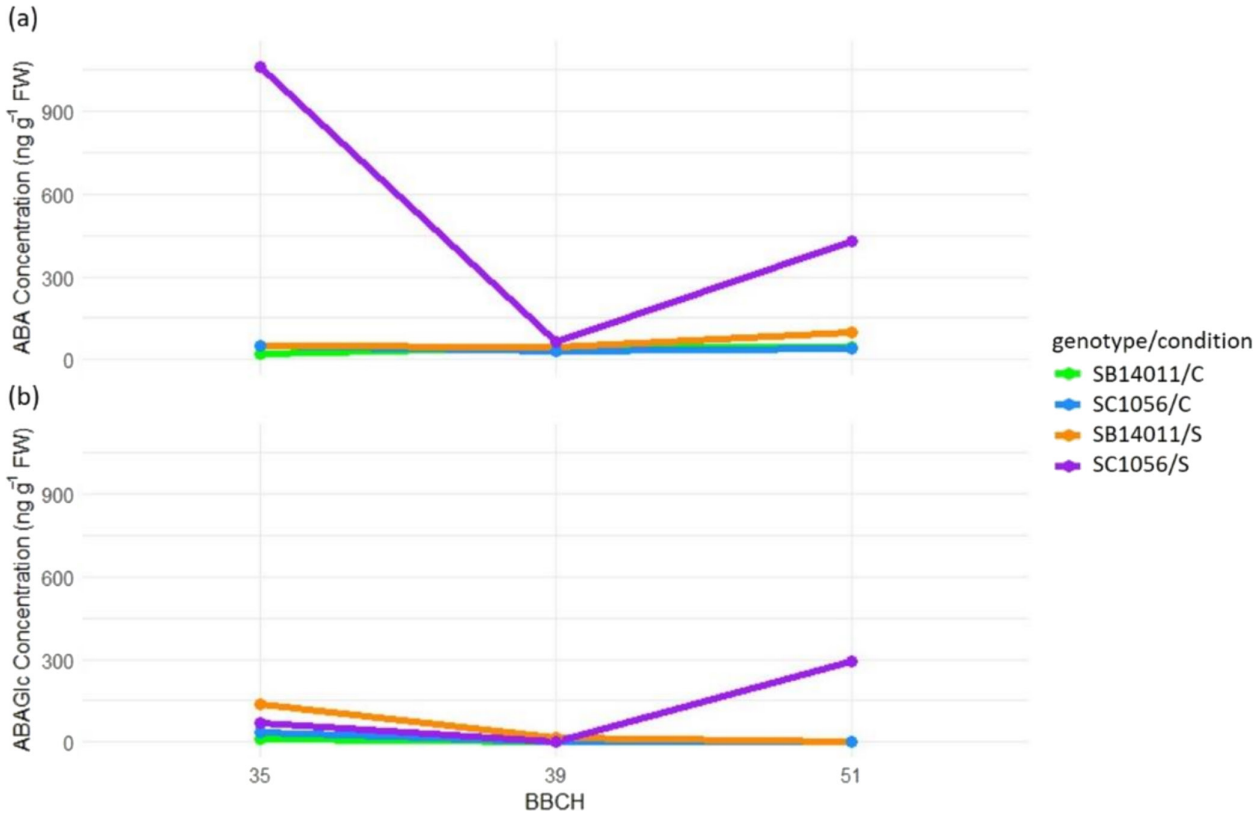

FIGURE 7 | Representation of genotype x condition x treatment interaction for abscisic acid (ABA) and abscisic acid glucose ester (ABAGe) concentration; treatment: BBCH35, BBCH39 and BBCH51; genotypes: the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056, conditions: optimal conditions (C), stress (S).

(correlations under optimal conditions are provided in the Supplementary section, Figure S1, S2). The following section describes only those correlations between yield, pollen traits, and phytohormones that are relevant for interpreting the cold stress response. Yield parameters include grain dry yield, panicle dry yield, and panicle harvest index, while pollen traits include the total number of fertile pollen and cell concentration.

3.4.1 | SB14011 (Cold-Tolerant)

Under stress conditions (Figure 11), the panicle harvest index (PHI) shows a strong negative correlation with all gibberellins (GA12, GA15, GA19, GA24, GA44, and GA53). Apart from that, no strong correlations were found between phytohormones and either yield or pollen traits.

Under optimal conditions, neither yield parameters nor pollen traits nor phytohormones were strongly correlated with each other (Figure S1).

3.4.2 | SC1056 (Cold-Sensitive)

Under stress conditions (Figure 12), yield parameters were strongly correlated with ABAGlc, JA, and JAIle. Pollen traits, however, showed no strong correlations with either yield parameters or phytohormones.

Under optimal conditions, pollen traits as well as JAIle were strongly negatively correlated with GA44, GA19, GA53, and total GA. A strong positive correlation was found between pollen traits and JAIle. Yield parameters showed no strong correlations with either pollen traits or phytohormones (Figure S2).

4 | Discussion

4.1 | Abscisic Acid and Its Derivatives

4.1.1 | Abscisic Acid

The presence of genes regulating the concentration of abscisic acid (ABA) and enabling cold-tolerant genotypes to produce lower amounts of ABA has been previously reported for rice (Sharma and Nayyar 2016). The elevated synthesis of ABA has been attributed to increased expression of the two biosynthetic ABA genes, *OSZEP1* and *OSNCED3*. When comparing with the cold-sensitive rice genotype, the cold-tolerant rice genotype exhibited lower expression of both genes, leading to the observed reduced ABA levels (Oliver et al. 2007; Ji et al. 2011). Our results confirm this phenomenon for sorghum as well. Here, the cold-tolerant inbred line SB14011 maintains a consistently low level of ABA under cold stress across all stages when compared to optimal growth conditions. Conversely, a significant increase in ABA concentration under cold stress at

TABLE 5 | Results of the ANOVA and subsequent Tukey test examining the phytohormone concentration of dihydrophaseic acid (DHPA) and phaseic acid (PA) at various developmental stages under control and stress conditions for the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056 (significance level: *** 0.001; ** 0.01.; *0.05).

		DHPA					PA				
SB14011	**ANOVA**	$p = 0.0218$ *					$p = 0.00291$				
Condition	**Contrasts**	**Estimate**	**SE**	**df**	**t.ratio**	***p***	**Estimate**	**SE**	**df**	**t.ratio**	***p***
Control	BBCH35-BBCH39	−285	90.7	15	−3.142	0.0067	/	/	/	/	/
Control	BBCH35-BBCH51	−168	90.7	15	−1.849	0.0842	/	/	/	/	/
Control	BBCH39-BBCH51	117	90.7	15	1.292	0.2158	/	/	/	/	/
SC1056	ANOVA			$p = 0.55$					$p = 0.239$		
Control	BBCH35-BBCH39	/	/	/	/	/	/	/	/	/	/
Control	BBCH35-BBCH51	/	/	/	/	/	/	/	/	/	/
Control	BBCH39-BBCH51	/	/	/	/	/	/	/	/	/	/
SB14011	ANOVA			0.00133					0.171		
Stress	BBCH35-BBCH39	/	/	/	/	/	/	/	/	/	/
Stress	BBCH35-BBCH51	/	/	/	/	/	/	/	/	/	/
Stress	BBCH39-BBCH51	/	/	/	/	/	/	/	/	/	/
SC1056	ANOVA			0.221					$p = 0.00053$ ***		
Stress	BBCH35-BBCH39	/	/	/	/	/	1524	375	15	4.066	0.001
Stress	BBCH35-BBCH51	/	/	/	/	/	1761	375	15	4.699	0.0003
Stress	BBCH39-BBCH51	/	/	/	/	/	237	375	15	0.633	0.5363

all stages (BBCH35, BBCH39, and BBCH51) can be confirmed in the cold-sensitive genotype SC1056. This could indicate that the ABA regulatory mechanisms observed in rice may also be transferable to sorghum. In rice, the presence of genetic sequences in the cold-tolerant line enables it to maintain a low ABA concentration, whereas the absence of such sequences in the cold-sensitive genotype leads to elevated ABA levels (Ji et al. 2011; Sharma and Nayyar 2016).

Pollen sterility has been discussed in several studies as the main reason for reduced cereal yields after cold stress. Various studies have shown that ABA levels can directly influence tapetal development and thus pollen fertility (Sakata et al. 2014). Sharma and Nayyar suggest that the ability to accumulate low ABA levels under cold stress contributes to making anthers more resistant to ensure the development of viable pollen. Increased ABA accumulation as a cause of impaired pollen fertility has been proposed in several studies. In sorghum and rice, meiosis and microspore together with tapetal development leading to male sterile pollen have been identified as particularly cold-sensitive (Downes and Marshall 1971; Mamun et al. 2006; Wood et al. 2006; Gothandam et al. 2007). Cold-tolerant rice plants are capable of further reducing ABA levels in the anthers through increased expression of ABA hydroxylation genes (Nambara and Marion-Poll 2005; Oliver et al. 2007). Here, ABA is converted into phaseic acid, thereby promoting ABA degradation. In contrast to the ABA genes, the expression of the two ABA 8-hydroxylase genes, *ABA8ox1* and *ABA8ox2*, is higher in cold-tolerant genotypes (Oliver et al. 2007; Li et al. 2011). Besides the ABA hydroxylation genes, the cell wall invertase gene also appears to play a key role in rice. In the tapetum, in addition to maintaining low ABA levels, the expression of the cell wall invertase gene (*INV4*) ensures the development of fertile pollen (Ji et al. 2011). In cold-sensitive genotypes, the suppression of the cell wall invertase gene by increased ABA accumulation appears to be the cause of abnormal pollen development. However, this mechanism does not seem to apply to all examined developmental stages in sorghum.

The cold-sensitive genotype used in a previous study conducted by us showed no significant reduction in pollen fertility at the cold-sensitive developmental stage BBCH39. Despite cold influence at this stage, pollen fertility remained at a level comparable to that of the control and post-cold stage BBCH51. Only cold stress from BBCH35 resulted in a significant reduction in fertile pollen, yet pollen fertility still exceeded 60%. A pollination experiment revealed that pollen fertility was not the determining factor for yield losses caused by cold stress (Neitzert et al. 2025). This conclusion is supported by the correlation analysis, which revealed no significant relationships between pollen traits and yield parameters—neither under optimal nor under stress conditions. This underscores that the observed yield losses are not primarily attributable to reduced pollen fertility. Based on this, it can be concluded that the significantly increased ABA concentration after cold stress at

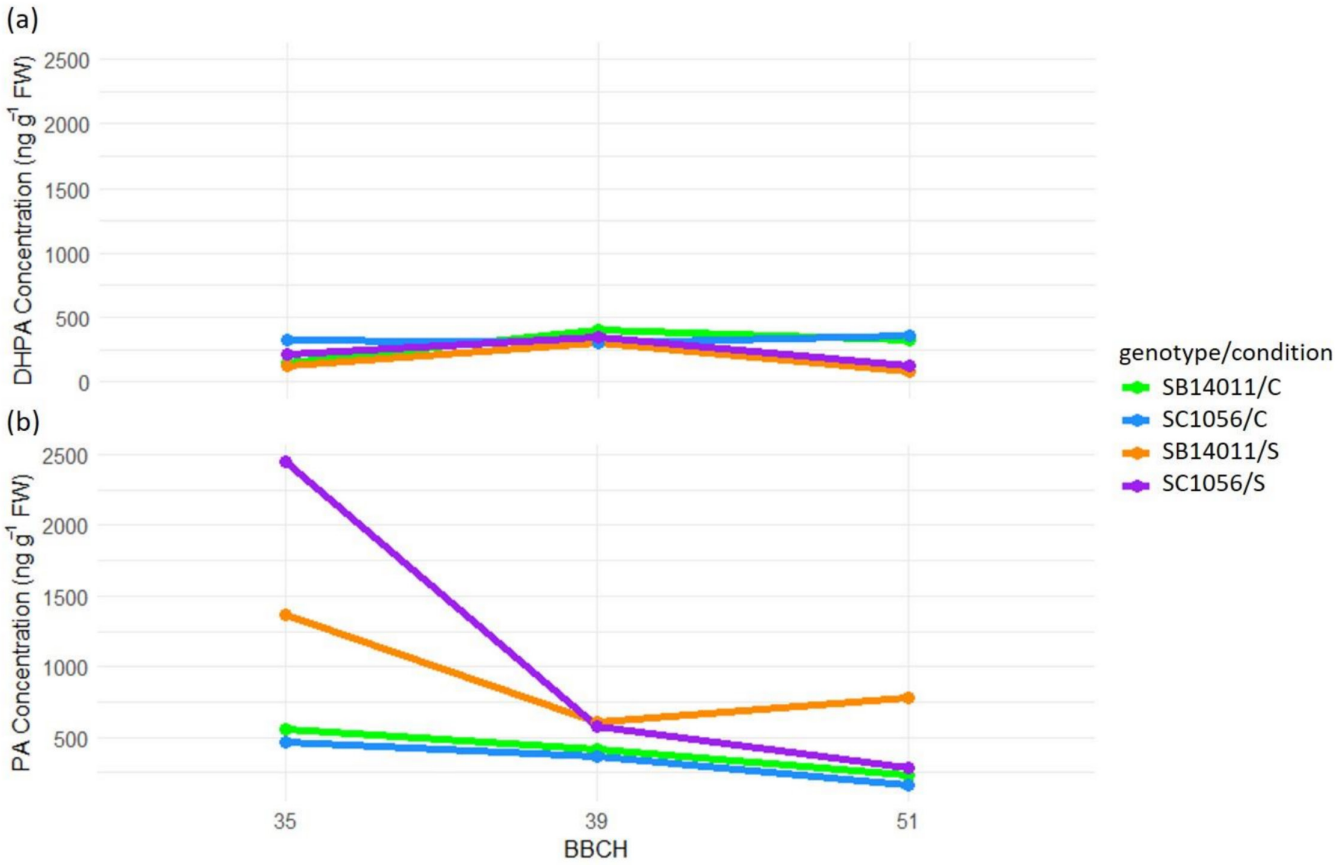

FIGURE 8 | Representation of genotype x condition x treatment interaction for DHPA and PA concentration; treatment: BBCH35, BBCH39 and BBCH51; genotypes: the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056, condition: optimal conditions (C), stress (S).

BBCH39 is not accompanied by a reduction in pollen fertility. Therefore, the theory that the ability to develop fertile pollen depends on the genotype's ability to maintain low ABA concentrations can only be applied for cold from BBCH35 onwards. As described in other cereal species, ABA 8′-hydroxylase and cell wall invertase may play an important role in controlling ABA homeostasis in the anthers and pollen development during the reproductive stage (Ji et al. 2011).

Contrary to reports in rice and other cereals, one explanation for the typically high pollen fertility despite elevated ABA accumulation after cold stress at BBCH39 in the cold-sensitive genotype could be impairment of the female floral organ. Impairment of the female floral organ can also be discussed for cold stress at BBCH35, as increased cold sensitivity of the female floral organ has already been observed in other plant species, leading to various impairments, such as irreversible abortion of the egg cell or delayed maturation and increased embryo mortality (Casper 1990; Srinivasan et al. 1999). Since the functionality of the egg cell is crucial for normal pistil operation (Dumas et al. 1984), this could indicate that cold also affects this function in sorghum. Further studies are required to precisely determine to what extent and in which phases of female gametogenesis the effects are most affected. Thus, sorghum would fundamentally differ from rice, where low ABA levels in the anthers are considered a key determinant of fertility. At the BBCH51 stage, cereals are in the transition between spikelet and floret development (Guo et al. 2015). Schaffasz et al. (2019) showed that sorghum is no longer susceptible to cold stress from BBCH stage 43 onward, as indicated by high pollen viability of over 80%. The resilience of the cold-tolerant genotype SB14011 after cold stress at the BBCH51 stage could be explained by the completed and more sensitive spikelet development. Cold stress and the resulting higher ABA levels appear to have no further impact on the development of the male or female floral organ at the onset of floret development, which aligns with the findings of Schaffasz et al. (2019).

When considering the cold-tolerant genotype, the ABA level remains consistently high across all developmental stages, but never at zero. This is because a certain amount of ABA is required for normal anther development and the ensuring of fertile pollen (Wang et al. 1999). For sorghum, the average ABA concentration in the cold-tolerant genotype after cold stress is 96.127 ng g^{-1} FW across all stages examined. In comparison, the average ABA concentration of the cold-sensitive genotype is five-fold higher (503.52 ng g^{-1} FW).

4.1.2 | Abscisic Acid Glucose Ester (ABAGe)

Looking at abscisic acid glucose ester (ABAGe), a conjugate of abscisic acid serving as the storage and transport form of the hormone ABA, no significant differences in ABAGe

TABLE 6 | Results of the ANOVA and subsequent Tukey test examining the phytohormone concentration of jasmonic acid (JA) and JA-isoleucine (JAIle) at various developmental stages under control and stress conditions for the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056 (Significance level: *** 0.001; ** 0.01.; *0.05).

		JA					JAIle				
SB14011	**ANOVA**	$p = 0.274$					$p = 0.00509$ **				
Condition	**Contrasts**	**Estimate**	**SE**	**df**	**t.ratio**	***p***	**Estimate**	**SE**	**df**	**t.ratio**	***p***
Control	BBCH35-BBCH39	/	/	/	/	/	1.73	25.3	15	0.068	0.9465
Control	BBCH35-BBCH51	/	/	/	/	/	−84.77	25.3	15	−3.356	0.0043
Control	BBCH39-BBCH51	/	/	/	/	/	−86.49	25.3	15	−3.424	0.0038
SC1056	ANOVA	$p = 0.135$					$p = 0.000202$ ***				
Control	BBCH35-BBCH39	/	/	/	/	/	−15.4	18	11	−0.855	0.4107
Control	BBCH35-BBCH51	/	/	/	/	/	−101.5	17	11	−5.977	0.0001
Control	BBCH39-BBCH51	/	/	/	/	/	−86.1	18	11	−4.78	0.0006
SB14011	ANOVA	0.0718					5.09e-07 ***				
Stress	BBCH35-BBCH39	/	/	/	/	/	−0.727	11.4	15	−0.064	0.9502
Stress	BBCH35-BBCH51	/	/	/	/	/	−93.578	11.4	15	−8.182	< 0.0001
Stress	BBCH39-BBCH51	/	/	/	/	/	−92.852	11.4	15	−8.118	< 0.0001
SC1056	ANOVA	$p = 5.69e\text{-}06$ ***					$p = 8.96e\text{-}06$ ***				
Stress	BBCH35-BBCH39	3.58	5.57	15	0.641	0.531	−2.04	10.5	15	−0.194	0.8489
Stress	BBCH35-BBCH51	−35.5	5.57	15	−6.368	< 0.0001	−69	10.5	15	−6.555	< 0.0001
Stress	BBCH39-BBCH51	−39.07	5.57	15	−7.01	< 0.0001	−66.96	10.5	15	−6.361	< 0.0001

concentration between control and stress conditions at sensitive stages (BBCH35 and BBCH39) could be observed in either the cold-tolerant or cold-sensitive genotype. Furthermore, the ABAGe concentration of both genotypes remains at a similar level during sensitive stages. When comparing individual developmental stages within each genotype, significant differences occur under cold stress. While SB14011 (cold-tolerant) exhibits significantly higher ABAGe concentrations in BBCH35 compared to BBCH51 after cold stress, SC1056 (cold-sensitive) shows significantly lower ABAGe concentrations in BBCH35 and BBCH39 compared to BBCH51. The amount of ABA accumulating in a plant is influenced not only by ABA production but also by processes such as ABA degradation and conjugation to other molecules (Ren et al. 2007). The observed increased accumulation of ABAGe in BBCH35 could be due to ABA regulation through conjugation. At the same time, the reduced amount of ABAGe in the sensitive developmental stages of the cold-sensitive genotype could be explained as a consequence of increased ABA release. Especially in BBCH35, where ABA peaked at 1729.8 ng g^{-1} FW after cold stress, a depleted ABAGe reservoir seems plausible. Under optimal growth conditions, the ABAGe levels of both genotypes remain consistent across all developmental stages, reinforcing the theory of the depleted ABAGe reservoir in SC1056 after cold exposure. The significant correlation of ABAGlc with yield parameters in the cold-sensitive genotype (SC1056) under cold stress indicates that the glucoside form of the hormone is directly linked to yield performance.

4.1.3 | Dihydrophaseic Acid and Phaseic Acid

Besides the previous inactivation of abscisic acid (ABA) through conjugation, ABA can also be deactivated through oxidation (Zeevaart 1999; Kushiro et al. 2004; Nambara and Marion-Poll 2005). In this oxidation process, phaseic acid (PA) and dihydrophaseic acid (DHPA) are breakdown products of ABA. The most common hydroxylation in ABA catabolism occurs at C-8′, generating 8'-Hydroxy-ABA, which later isomerizes to PA. DHPA is produced through further reduction of PA. At this point, alongside DHPA, the analog epi-DHPA can also form (Krochko et al. 1998; Cutler and Krochko 1999). In cold-tolerant rice, it has been demonstrated that the expression of ABA 8-hydroxylase genes, which convert ABA to phaseic acid, was significantly higher than in cold-sensitive varieties (Oliver et al. 2007; Ji et al. 2011).

In this study, after cold stress, PA is elevated in both tolerant and susceptible genotypes. This implies that the expression of ABA 8-hydroxylase genes seems to be high regardless of genotype-specific cold tolerance levels. While the comparison between growth under optimal conditions and under cold stress reveals that the cold-tolerant line exhibits only an increased PA concentration in BBCH35, the PA concentration is significantly elevated in both sensitive stages (BBCH35 and BBCH39) in the cold-sensitive genotype.

Therefore, the cold-sensitive genotype appears to utilize oxidation instead of conjugation (see Section 4.1.2) to counteract

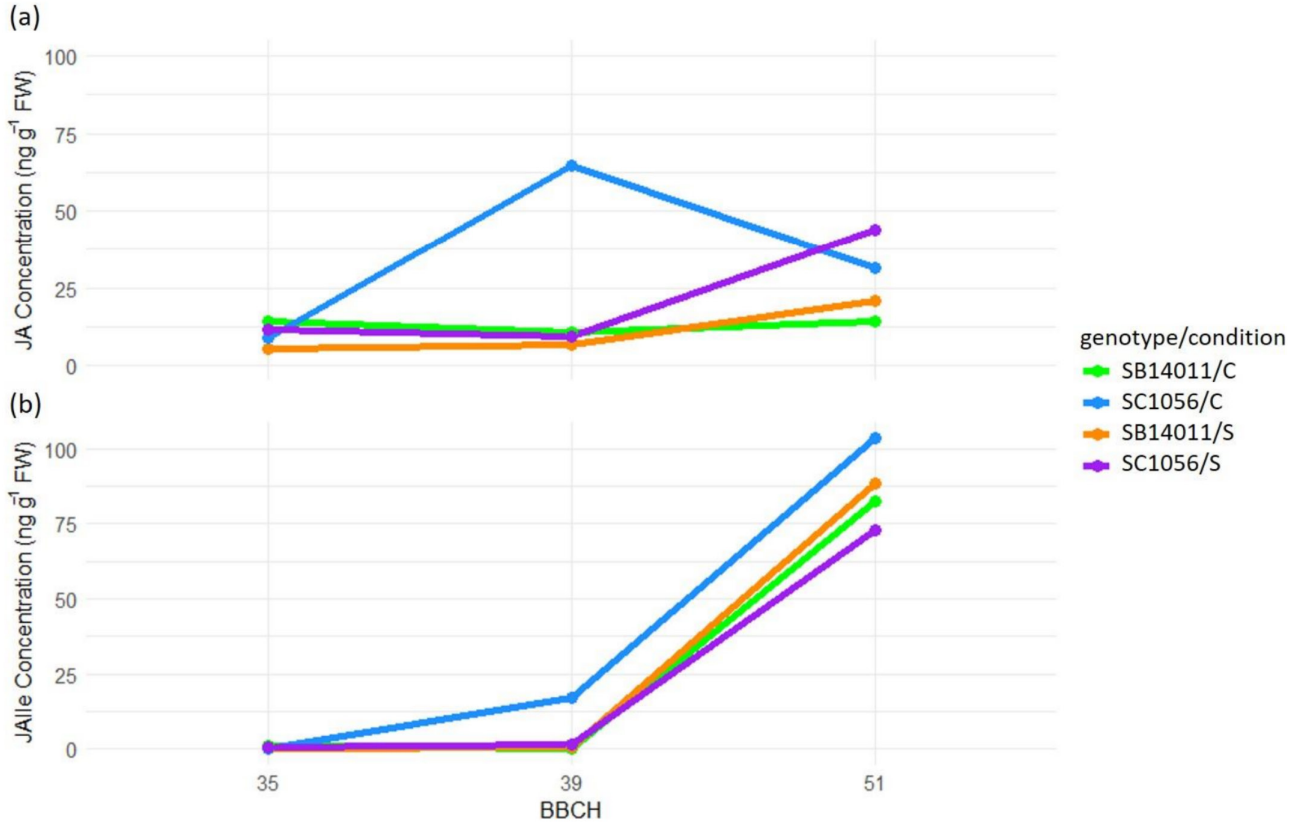

FIGURE 9 | Representation of genotype x condition x treatment interaction for JA and JAIle concentration; treatment: BBCH35, BBCH39 and BBCH51; genotypes: the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056, condition: optimal conditions (C), stress (S).

the significantly increased ABA concentrations in stages sensitive to cold. The elevated ABAGe (Section 4.1.2) and PA levels in the cold-tolerant genotype suggest that it utilizes both conjugation and oxidation pathways to deactivate ABA. This indicates that oxidation and conjugation pathways are active under cold stress. However, while SB14011 shows consistent PA concentrations between developmental stages after cold stress, SC1056 exhibits significantly higher PA levels in BBCH35 compared to BBCH39 and BBCH51. This can be explained by the extremely high accumulation of ABA in this developmental stage. The generally high PA contents compared to ABA and DHPA suggest that PA has a long half-life and is metabolized relatively slowly to DHPA.

In contrast, in other plant species, degradation of ABA predominates over PA or DHPA (Balsevich et al. 1994; Setha et al. 2005; Huang et al. 2008). These findings suggest that, as described by Garbero et al. (2011), ABA metabolism is regulated differently in various plant species. The data suggest that sorghum maintains ABA homeostasis through concurrently active conjugation (ABAGe) and oxidation pathways (PA/DHPA). In contrast to rice, where increased ABA oxidation to PA is associated with cold tolerance, both genotypes examined here show comparable PA accumulation. This indicates that the activity/expression of ABA 8-hydroxylase genes in sorghum is not a genotype-specific limiting factor—at least in the genotypes, developmental stages, and conditions tested.

4.2 | Gibberellins

Cold stress in rice causes a reduction in the endogenous levels of bioactive gibberellins. Cold-tolerant rice genotypes capable of maintaining a sufficient pool of bioactive gibberellins under cold stress through increased gibberellins (GA) catabolism have been identified (Sharma and Nayyar 2016). Problems arise with hypertrophy of tapetal cells and pollen production in cold-sensitive genotypes with insufficient levels of GA. Suppression of the cell wall-bound acid invertase gene and the monosaccharide transporter gene disrupts sugar transport to the tapetum, leading to abnormal pollen development (Oliver et al. 2005; Mamun et al. 2006).

Both the cold-tolerant and sensitive sorghum genotypes exhibited significant differences between optimal conditions and cold stress. Both responded equally sensitively to cold in BBCH39 with reduced GA concentrations. Maintaining the GA pool in BBCH35 and its decline in BBCH39, along with a reduced grain count (Neitzert et al. 2025), could suggest that maintaining a sufficiently high pool of GAs is less relevant for the ability to achieve full grain formation after reproductive cold stress, similar to the suppression of ABA production. Additionally, this supports the assumption that pollen fertility in sorghum is less restricted by cold and rather involves the sensitivity of the female reproductive organ (Osuna-Ortega et al. 2003). In addition to the high pool of active gibberellins in both genotypes after cold stress in BBCH35, the high pollen fertility in the cold-tolerant genotype after stress in sensitive stages (BBCH35 and BBCH39)

TABLE 7 | Results of the ANOVA analysis and subsequent Tukey test examining the phytohormone concentration of gibberellins (GA) at various developmental stages under control and stress conditions for the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056 (significance level: *** 0.001; ** 0.01.; *0.05).

		GA				
SB14011	**ANOVA**	***p* = 4.94e-12 ***				
Condition	**Contrasts**	**Estimate**	**SE**	**df**	**t.ratio**	***p***
Control	BBCH35-BBCH39	54.53	3.2	15	17.045	< 0.0001
Control	BBCH35-BBCH51	64.14	3.2	15	20.047	< 0.0001
Control	BBCH39-BBCH51	9.61	3.2	15	3.002	0.0089
SC1056	ANOVA			*p* = 0.0128 *		
Control	BBCH35-BBCH39	23.33	9.41	11	2.480	0.0306
Control	BBCH35-BBCH51	31.28	8.87	11	3.526	0.0048
Control	BBCH39-BBCH51	7.95	9.41	11	0.844	0.4165
SB14011	ANOVA			*p* = 0.00084 ***		
Condition	Contrasts	Estimate	SE	df	t.ratio	*p*
Stress	BBCH35-BBCH39	65.252	15.6	15	4.177	0.0008
Stress	BBCH35-BBCH51	6.077	15.6	15	4.230	0.0007
Stress	BBCH39-BBCH51	0.825	15.6	15	0.053	0.9586
SC1056	ANOVA			*p* = 5.46e-05 ***		
Stress	BBCH35-BBCH39	52.72	9.52	15	5.540	0.0001
Stress	BBCH35-BBCH51	52.21	9.52	15	5.486	0.0001
Stress	BBCH39-BBCH51	−0.51	9.52	15	−0.054	0.9580

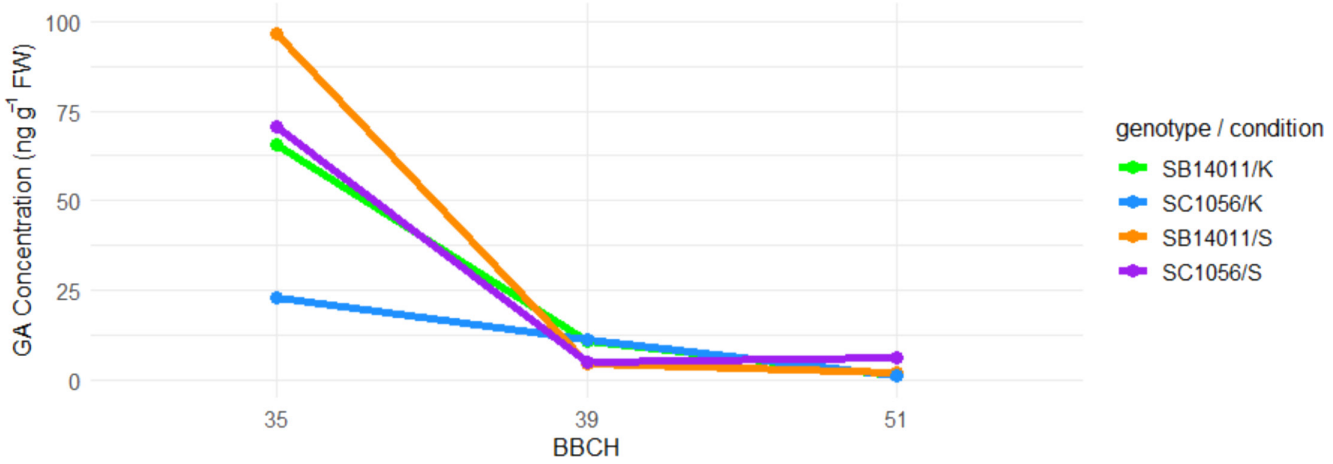

FIGURE 10 | Representation of genotype x condition x treatment interaction for the gibberellin concentration; treatment: BBCH35, BBCH39 and BBCH51; genotypes: the cold-tolerant genotype SB14011 and the cold-sensitive genotype SC1056, condition: optimal conditions (C), stress (S).

despite a decline in GAs in BBCH39 contradicts the assumption that, as in rice, a reduction in GAs leads to abnormal pollen development. Furthermore, the correlation analysis showed that pollen traits were not correlated with yield parameters, supporting the conclusion that yield sensitivity to cold in sorghum is not driven by limitations in pollen development but rather by other, presumably female reproductive processes.

When comparing GA levels between the two genotypes, regardless of condition and stress, no differences were observed after exposure to cold. This suggests that the response of gibberellin synthesis to cold is very similar in both genotypes. Significant differences were observed in GA concentration between developmental stages. Here, the same trend is observed regardless of genotype and condition (stress and optimal conditions). In the

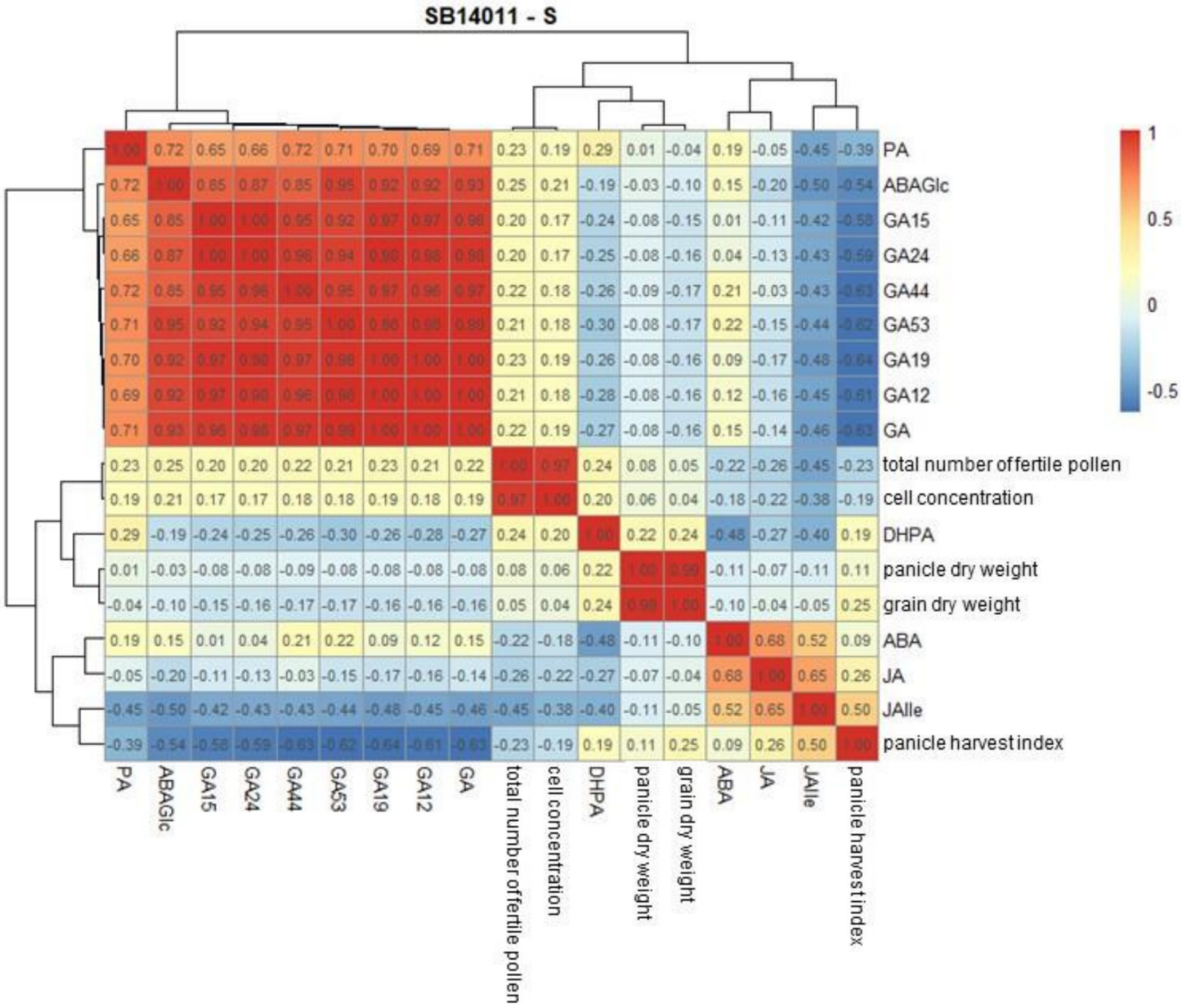

FIGURE 11 | Heatmap illustrating Pearson's correlations among the analyzed traits for the cold-tolerant genotype SB14011 under cold stress: abscisic acid (ABA), abscisic acid glucose ester (ABAGe), dihydrophaseic acid (DHPA), jasmonic acid (JA), phaseic acid (PA), gibberellins (GA) and their derivatives (GA12, GA15, GA19, GA24, GA44, and GA53), total number of fertile pollen, cell concentration, panicle harvest index, panicle dry weight, and grain dry weight. Strong positive correlations (r = 1) are shown in red, while strong negative correlations (r = −1) are shown in blue.

sensitive stage BBCH35, GA concentration is highest in all cases, with a continuous decline in concentration towards BBCH51 (details in Section 4.4). This suggests that GA bottlenecks in sorghum are not the limiting factor for reproductive cold tolerance, and that breeding strategies aimed at stabilizing GA pools are likely less effective in sorghum than in rice.

Against this background, cold-tolerant rice genotypes are known to maintain an adequate pool of bioactive gibberellins under cold stress, in part via altered biosynthetic pathways of GA4 and GA7 (Sharma and Nayyar 2016). In sorghum, however, the presence of GA4 and GA7 could not be confirmed in this experiment under either optimal conditions or after cold stress. Together with the similar GA profiles of the two genotypes examined, this supports the view that GA-related bottlenecks are not the decisive factor for reproductive cold tolerance in sorghum.

4.3 | Jasmonic Acid and Jasmonic Acid Isoleucine

Jasmonic acid (JA) and its biologically active form jasmonic acid isoleucine (JAIle) have been linked to cold stress in several studies. As a signaling molecule for stress responses, increased jasmonic acid production can prepare the plant for stress and is involved in the regulation of stress responses. In grasses, JA has been found to play a significant role in the development of generative tissue (Cai et al. 2014). The ICE-CBF transcriptional regulation pathway has been identified as particularly important in maintaining plant development under cold stress conditions (Kim et al. 2015). In Arabidopsis, it has been demonstrated that the biosynthesis of the phytohormone regulates the expression of cold-responsive genes through the CBF transcriptional pathway, which are responsible for improved cold tolerance (Hu et al. 2017). Further studies have shown that in rice, several genes related to JA biosynthesis (including *AOC*, *AOS1*, *AOS2*,

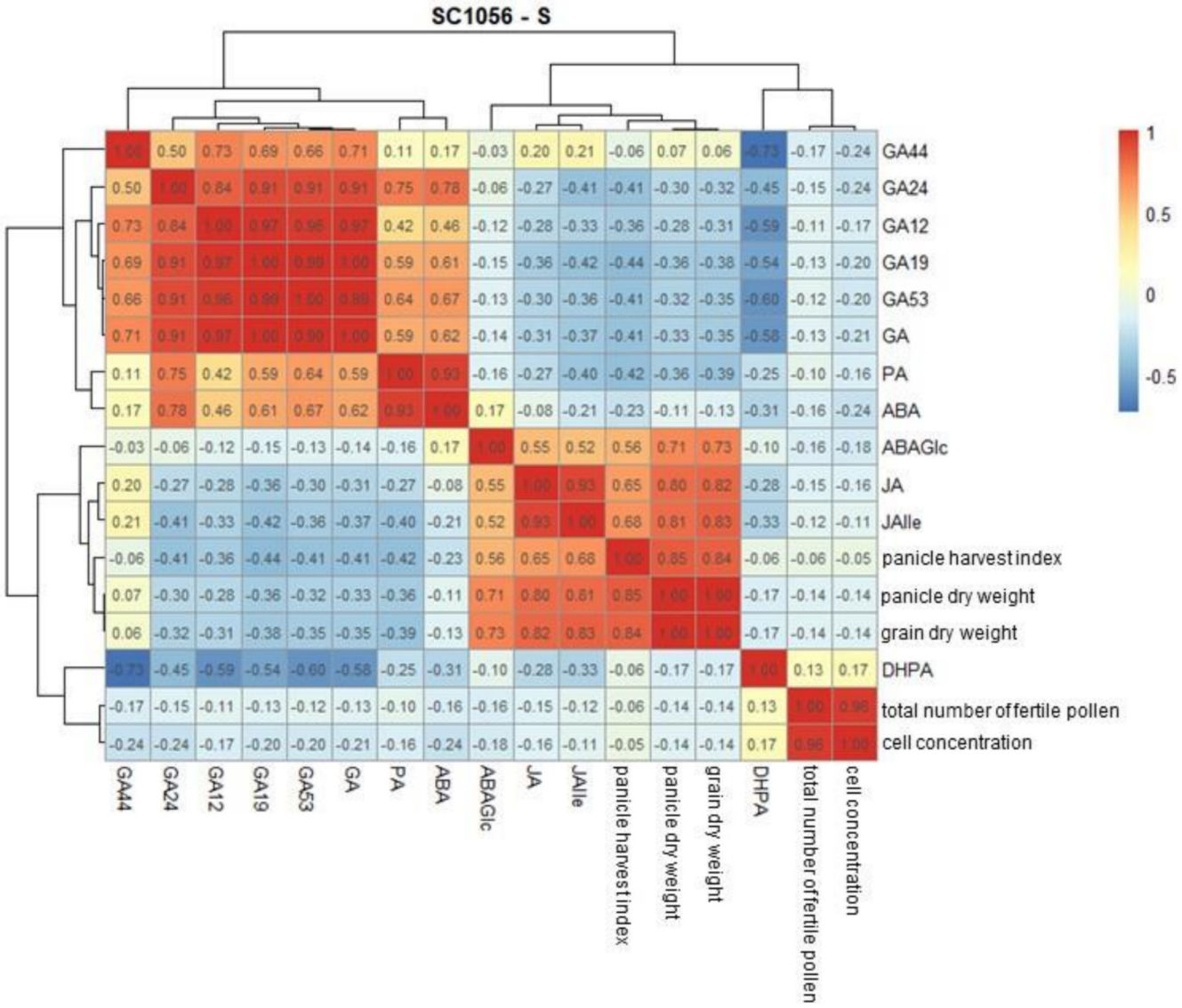

FIGURE 12 | Heatmap illustrating Pearson's correlations among the analyzed traits for the cold-sensitive genotype SC1056 under cold stress: abscisic acid (ABA), abscisic acid glucose ester (ABAGe), dihydrophaseic acid (DHPA), jasmonic acid (JA), phaseic acid (PA), gibberellins (GA) and their derivatives (GA12, GA15, GA19, GA24, GA44, and GA53), total number of fertile pollen, cell concentration, panicle harvest index, panicle dry weight, and grain dry weight. Strong positive correlations (r = 1) are shown in red, while strong negative correlations (r = −1) are shown in blue.

and *LOX2*), as well as signaling genes (*COI1a* and *bHLH148*), respond positively to cold stress (Du et al. 2013; Hu et al. 2017). Furthermore, jasmonic acid plays a role in protecting against pests and diseases by inducing defense mechanisms, which may help protect the plant during the cold tolerance process (Chini et al. 2016; Zhang et al. 2017; Al-Zahrani et al. 2020). An increased production of jasmonic acid by pests is excluded in this experiment due to controlled conditions in the climate chamber experiment.

In this study, no significant differences were observed in the concentration of JA and JAIle between the two genotypes under optimal conditions and cold stress across all developmental stages. Also, no differences were observed between the genotypes in the sensitive stages (BBCH35 and BBCH39), regardless of condition. Contrary to expectations, based on these results, it can be summarized that the accumulation of jasmonic acid in cold stress in sorghum seems to be low compared to other grasses (Cai et al. 2014; Du et al. 2013). With the absence of increased JA accumulation, the expression of JA biosynthesis genes at low temperatures in sorghum, unlike in rice (Du et al. 2013), does not seem to be induced by cold. To test this hypothesis, quantitative gene expression analyses (qPCR) or transcriptome analyses would be useful to determine whether JA biosynthesis genes are indeed unresponsive to cold in sorghum.

However, when comparing between developmental stages after cold stress, the cold-sensitive genotype shows significantly lower JA concentrations in stages BBCH35 and BBCH39 compared to BBCH51. In contrast, the cold-tolerant genotype maintains a consistent level of JA across all stages investigated. It can be assumed that there is generally a sufficient level of JA in sorghum to control the processes necessary for maintaining

plant development under cold stress (Kim et al. 2015). Also, concerning the concentration of JAIle, significant differences between developmental stages exist. Here, regardless of genotype and condition (stress and optimal conditions), the same trend is observed. In the sensitive stages BBCH35 and BBCH39, the concentration of JAIle is lower in all cases than in BBCH51, with a continuous increase with the plant's age. The consistent trend of JAIle in the cold-tolerant genotype and the absence of differences from optimal conditions to cold stress support the assumption that there seems to be a sufficiently high level of jasmonic acid and its derivatives in sorghum to ensure plant development under cold conditions. However, this does not provide an explanation for the lack of differences between cold-tolerant and cold-sensitive genotypes. In addition to JAIle, the derivative methyl jasmonate (MeJA) can also be formed in the cytoplasm from jasmonic acid. MeJA has already been associated with reduced cold damage in *Curcurbita pepo L*. (Wang and Buta 1994). In the future, investigating the MeJA derivative could provide insights into the role of JA and its derivatives.

4.4 | JA-GA Cross-Talk

Jasmonic acid (JA), the defense signal, and gibberellins (GA), the hormone required for growth, interact in the form of antagonistic or synergistic influence in several signaling pathways. This allows the plant to adjust growth and adaptation to the prevailing environmental conditions. Several studies have shown that there is an interaction between JA and GA under both normal and stress conditions (Achard et al. 2008; Um et al. 2018).

In *Arabidopsis thaliana*, it has been shown that under normal conditions, accumulation of GA leads to the degradation of DELLA proteins. Under cold stress, the GA level is reduced, leading to an accumulation of DELLA proteins (Achard et al. 2008). DELLA proteins activate by binding to JAZ proteins, which regulate jasmonate-responsive genes (Wingler et al. 2020).

Our results suggest the existence of an antagonistic interaction between JAIle, the biologically active form of JA, and GA concentration in Sorghum. While the JAIle concentration increases with the age of the plant regardless of whether the plant is exposed to optimal conditions or stress, the GA concentration decreases progressively, reaching its peak at the beginning of the early reproductive phase. This is evidence that antagonistic JA-GA crosstalk occurs in Sorghum under both control and stress conditions. However, the extent to which this cross-talk regulates plant growth and development under stress conditions needs to be further elucidated in additional experiments.

5 | Outlook

The results of this study lay a foundation for elucidating the physiological mechanisms underlying reproductive cold tolerance in sorghum. The focus was on analyzing phytohormone dynamics under cold stress.

A limiting factor of this study is the number of biological replicates: for each treatment and developmental stage, three independent plants were available, each analyzed in two technical replicates to increase measurement precision. A higher number of biological replicates would further strengthen the statistical power. Nevertheless, the results provide robust insights into hormonal dynamics during the reproductive cold stress response and form a solid basis for further investigations.

To gain a more comprehensive understanding of the mechanisms underlying cold tolerance, additional approaches are necessary. Transcriptome analyses, for example, could provide insights into the molecular basis, while physiological measurements such as flag leaf photosynthetic activity or chlorophyll content could help link hormonal responses to physiological adaptations. The observed differences in ABA, GA, and JA dynamics provide valuable hypotheses for future studies in which hormone profiling, gene expression data, and physiological measurements are integrated.

Hormone profiles or the expression of hormone-related genes may serve as indirect selection markers—particularly at early developmental stages—potentially accelerating selection in practical breeding programs.

6 | Conclusion

A deeper understanding of reproductive cold tolerance is crucial for expanding sorghum cultivation into temperate regions. This study demonstrates that cold-tolerant sorghum is capable of downregulating ABA concentration under cold stress. Contrary to the literature on rice, which often links elevated ABA levels and insufficient pools of bioactive gibberellins in sensitive genotypes to abnormal pollen development, this does not appear to be the case in sorghum. Additionally, an antagonistic interaction between GA and JA was observed, independent of genotype and environmental conditions. These findings contribute to a better understanding of the physiological mechanisms of cold tolerance in sorghum and may provide valuable insights for future breeding efforts aimed at accelerating the spread of cold-tolerant sorghum varieties in temperate climates.

Author Contributions

L.N. planned and supervised the climate chamber experiments and data collection, performed the data analysis, interpreted the results, and wrote the manuscript. N.K. contributed to data analysis. Y.A.T.M. and N.W. carried out the phytohormone analyses and contributed to manuscript editing. R.S. received the funding, contributed to the study design, and edited the manuscript. B.W. received the funding, interpreted the results, contributed to the study design, and edited the manuscript. S.W. designed the study and edited the manuscript. All authors contributed to the article and approved the submitted version.

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The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are provided in the Supplementary Material of this article.

Peer Review

The peer review history for this article is available in the Supporting Information for this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Heatmap illustrating Pearsons correlations among the analyzed traits for the cold-tolerant genotype SB14011 under optimal conditions: Abscisic acid (ABA), Abscisic acid glucose ester (ABAGe), Dihydrophaseic acid (DHPA), Jasmonic acid (JA). **Figure S2:** Heatmap illustrating Pearsons correlations among the analyzed traits for the cold-sensitive genotype SC1056 under optimal conditions: Abscisic acid (ABA), Abscisic acid glucose ester (ABAGe), Dihydrophaseic acid (DHPA), Jasmonic acid (JA). **Data S1:** Peer Review. **Data S2:** Supplementary Information.

5 Diskussion

5.1 Sorghum in gemäßigten Regionen – Potenzial und Herausforderungen

Der Klimawandel macht die verstärkte Anpassung von Nutzpflanzen an verschiedene abiotische Stressfaktoren unumgänglich (Raza et al. 2019). In den gemäßigten Breiten kommt es neben den Extremwetterereignissen zu zunehmend heißeren Sommern mit länger anhaltenden Dürreperioden und steigenden Niederschlagsmengen im Winter (Felsche et al. 2024). Diese Entwicklung unterstreicht die Notwendigkeit der Nutzung und Züchtung an die Klimawandelfolgen angepasster Kulturpflanzen. *Sorghum bicolor*, als eine aus den semiariden Gebieten stammende Kulturpflanze, ist optimal an Dürre angepasst. Die hohe Wasser- und Nährstoffnutzungseffizienz sowie die vielseitige Verwendbarkeit der tropischen C4-Pflanze birgt großes Potenzial für die Nutzung in den gemäßigten Regionen (Smith 2000; Tari et al. 2013).

Mit dem deutlich geringeren Wasserverbrauch bietet der Anbau von Sorghum vor allem in trockenen Gebieten eine umweltfreundliche und lukrative Alternative zu Mais (DMK Broschüre 2019). Wie ursprünglich auch Mais ist Sorghum seiner Herkunft entsprechend empfindlich gegenüber Kälte, was die Notwendigkeit der Züchtung kältetoleranter Sorten deutlich macht. Die erfolgreiche Adaptation von Mais als ebenfalls ursprünglich tropischer C4-Pflanze an kühlere klimatische Bedingungen kann dabei als Vorbild dienen (Reif et al. 2005).

Um die Anpassung an die gemäßigten Breiten zu ermöglichen, ist ein physiologisches Verständnis der Mechanismen, welche der Kältetoleranz von Sorghum zugrunde liegen, von zentraler Bedeutung. Die nachfolgenden Kapitel diskutieren (I) die Empfindlichkeit von Sorghum gegenüber Kälte in unterschiedlichen reproduktiven Entwicklungsstadien, (II) die Auswirkungen auf männliche und weibliche Blütenorgane, (III) phytohormonelle Veränderungen, (IV) genetische und züchterische Perspektiven sowie (V) zukünftige Forschungsperspektiven im Hinblick auf die Ergebnisse von Kapitel 3 und 4 (Neitzert et al. 2025 und 2026).

5.2 Geringer Kornansatz unter Kältestress – Ist die Pollenfertilität der limitierende Faktor?

Bei dem Anbau in den gemäßigten Breiten wird der Ertrag von Sorghumhirse maßgeblich durch Kälte begrenzt (Pinthus und Rosenblum 1961). Die tropische Kulturart ist ihrer Herkunft entsprechend an warme Temperaturen angepasst (Reshma et al. 2019). Einige Genotypen

sind durch den schrittweisen Anbau und nachfolgende Selektion in höher gelegene Regionen an kältere Temperaturen angepasst (Singh 1985).

Die Kältetoleranz von Sorghum wird in zwei unterschiedliche Phasen unterteilt, welche getrennt voneinander vererbt werden: die juvenile und die reproduktive Phase (Singh 1985). Während die juvenile Kältetoleranz bereits gut erforscht ist und mehrere an der Toleranz beteiligte QTL identifiziert sind, weiß man über die Kältetoleranz im reproduktiven Stadium weitaus weniger. Kälte im reproduktiven Stadium wirkt sich jedoch maßgeblich auf den Kornertrag aus. In Abhängigkeit der reproduktiven Stresstoleranz des Genotyps und der Stressintensität kommt es zu einer verminderten oder vollständig ausbleibenden Einkörnung (Maulana und Tesso 2013; Chopra et al. 2017; Emendack et al. 2021). Brooking (1976) beschreibt die Phase vor und während der Leptotänphase bzw. während der Mikrosporogenese bei Sorghumhirse als am empfindlichsten. Die Leptotän-Phase fällt etwa mit dem morphologischen Entwicklungsstadium BBCH37–BBCH39 zusammen, d. h. das Fahnenblatt ist vollständig entwickelt. Das konnte auch in der Studie von Neitzert et al. (2025) bestätigt werden. Hier reagierte der kälteempfindliche Genotyp nach Kälteeinwirkung in den frühen generativen Stadien (BBCH32, BBCH35 und BBCH39) am empfindlichsten auf den Stress. Auch bei Reis wurde das frühe Entwicklungsstadium der Mikrosporogenese als besonders empfindlich gegenüber Kältestress identifiziert (Satake und Havase 1970). Nach dem Beginn des Ährenschiebens nimmt die Empfindlichkeit von Sorghum laut beiden Studien ab. Zusätzlich zeigte sich, dass früherer und länger anhaltender Stress mit einer Verringerung der Rispenlänge einhergeht (Neitzert et al. 2025). Eine Abnahme der Rispenlänge nach Kältestress konnte auch bei kälteempfindlichen Reispflanzen beobachtet werden, bis zu einer Verringerung um 51,5 % (Bala et al. 2025; Kisara et al. 2025). Das zeigt, dass die geringen Erträge nicht nur aus einem verminderten Kornansatz resultieren, sondern auch durch kürzere Rispen verursacht werden. Als Ursache für den geringen Kornansatz führen Downes und Marshall (1971) eine verringerte Pollenfertilität an. Dies bestätigte auch Brooking (1976) anhand von mikroskopischer Pollenanalyse. Neben der verminderten Pollenfertilität wird auch eine Abnahme der Pollenmenge als Reaktion auf niedrige Temperaturen beschrieben (Brooking 1976; Ortega et al. 2003). Entgegen dieser Beobachtung gingen in der Studie von Neitzert et al. (2025) geringere Kornerträge nicht zwingend mit einer verminderten Pollenfertilität einher. Lediglich bei Kältestress ab BBCH35 wurde eine reduzierte Gesamtzahl fertiler Pollen beobachtet. Zusätzlich konnten eine erhöhte Zellkonzentration und eine

erhöhte Gesamtzahl fertiler Pollen beim kältetoleranten Genotyp und den reziproken Hybriden in einigen der empfindlichen Stadien beobachtet werden. Eine verstärkte Produktion fertiler Pollen bei kältetoleranten im Vergleich zu empfindlichen Pflanzen wurde bereits berichtet (Sharma und Nayyar 2016). Dass die Pollensterilität nicht zwingend der Grund für Ertragsminderungen nach Kältestress bei Sorghumhirse ist, wurde zusätzlich durch Bestäubungsexperimente bekräftigt. Mit dem Fokus auf den Samenertrag unterschieden sich die gestressten Pflanzen nicht voneinander, unabhängig davon, ob sie selbstbestäubt oder von einem ungestressten Pollenspender fremdbestäubt wurden. Dies ließ sich sowohl für den kältetoleranten als auch für den empfindlichen Genotyp beobachten (Neitzert et al. 2025). Dies legt nahe, dass anstelle der männlichen Blütenorgane vielmehr andere Faktoren den Kornertrag begrenzen. Osuna-Ortega et al. (2003) und Thakur et al. (2010) diskutierten die Rolle des weiblichen Blütenorgans bei der Kältetoleranz von Sorghum. Eine eingeschränkte Rezeptivität wurde als Ursache für den verminderten Kornansatz bzw. Kornertrag genannt. Auch bei anderen abiotischen Stressfaktoren wie Hitzestress wurde zunächst der Pollen als empfindlich identifiziert, während sich später die Rezeptivität des Pistils als gleichermaßen verantwortlich herausstellte (Djanaguiraman et al. 2018). Neuere Erkenntnisse zeigen ebenfalls, dass die Narben von Perlhirse und Sorghum in ähnlichem Maße auf Hitzestress reagieren und gleichermaßen empfindlich sind (Jagadish 2020). Selbst unter Trockenstress konnte gezeigt werden, dass die männliche Komponente nicht primär für den geringen Ertrag verantwortlich ist. Nach der Exposition gegenüber Wassermangel wurde zwar ein Anstieg steriler Pollen (bis zu 9 %) beobachtet, dies hatte jedoch keinen signifikanten Einfluss auf die Kornbildung der Sorghumpflanzen (Manjarrez-Sandoval et al. 1989).

Zusammenfassend deutet diese Studie darauf hin, dass die Pollenfertilität nicht der limitierende Faktor für den Samenansatz nach Kältestress bei Sorghum ist. Vielmehr könnte das weibliche Blütenorgan nach Kälteexposition beeinträchtigt sein (Neitzert et al. 2025). Dies deckt sich mit den zuvor beschriebenen Reaktionen von Sorghum auf andere abiotische Stressfaktoren wie Hitzestress (Djanaguiraman et al. 2018). Darüber hinaus wurde eine erhöhte Empfindlichkeit des weiblichen Gametophyten gegenüber Kältestress auch bei anderen Arten beobachtet – beispielsweise bei *Cryptantha flava* in Form von irreversiblem Eizellenabort (Casper 1990) oder bei Kichererbsen in Form von verzögerter Eizellenreifung und vermehrtem Embryoabort (Srinivasan et al. 1999). Da die Lebensfähigkeit der Eizelle für die normale Funktion des Stempels essenziell ist (Gaget et al. 1984), kann angenommen werden,

dass Kältestress auch diesen Aspekt bei den untersuchten Sorghum-Genotypen beeinflusst. Um zu verstehen, inwieweit das weibliche Blütenorgan durch Kältestress bei Sorghum beeinträchtigt wird, müssen weitere Versuche erfolgen.

5.3 Ein Blick in die Tiefe – Was passiert auf hormoneller Ebene?

Physiologische Strategien ermöglichen es Pflanzen, abiotischen Stress zu überstehen. Für die Steuerung dieser Stressstrategien ist unter anderem ein fein abgestimmtes Netzwerk aus Phytohormonen verantwortlich. Sich bestmöglich an veränderte Umweltbedingungen anpassen zu können, hängt also von der Fähigkeit der Pflanze ab, geeignete physiologische und hormonelle Regulationsmechanismen aktivieren zu können. Daher ist es entscheidend, Stresswahrnehmung, Signalweiterleitung und Anpassungsreaktionen zu entschlüsseln, um stresstolerante, ertragreiche Sorten zu entwickeln (Waadt et al. 2022; Das et al. 2025).

Welche hormonellen Mechanismen bei Sorghumhirse im reproduktiven Stadium zur Kältetoleranz beitragen, ist bisher ungeklärt. Mit Blick auf andere verwandte Kulturpflanzen konnten bei Reis Abscisinsäure (ABA) und Gibberelline (GA) als Schlüsselhormone bei Kältestress identifiziert werden (Oliver et al. 2007; Ji et al. 2011; Parish et al. 2012; Sharma und Nayyar 2016). ABA fungiert in Pflanzen als ein zentrales Stress- und Dormanzhormon, GA als Wachstums- und Keimungshormone. Beide Hormonsysteme agieren häufig antagonistisch (Lin et al. 2015; Shu et al. 2018).

In Bezug auf ABA konnte gezeigt werden, dass kältetolerante Reispflanzen niedrige ABA-Spiegel aufrechterhalten konnten, während die ABA-Konzentration in kälteempfindlichen Pflanzen deutlich höher war (Oliver et al. 2007). Auch bei Sorghumhirse zeigt sich dieser Trend. Über alle untersuchten empfindlichen Stadien hinweg hält der kältetolerante Genotyp unter Kältestress einen konstant niedrigen ABA-Spiegel. Umgekehrt zeigen sich im kälteempfindlichen Genotyp unter Stress signifikant höhere ABA-Konzentrationen (Neitzert et al. 2026). Die Fähigkeit, den ABA-Spiegel niedrig zu halten, wurde in Reis durch das Vorhandensein ABA-regulierender genetischer Sequenzen (OSZEP1 und OSNCED3) erklärt, welche die kälteempfindlichen Genotypen nicht aufweisen (Oliver et al. 2007; Ji et al. 2011; Sharma und Nayyar 2016). Außerdem wird angenommen, dass eine erhöhte ABA-Degradation durch Konjugation oder Oxidation in kältetoleranten Reispflanzen zur Reduktion der ABA-Spiegel beiträgt. Hier ist die Expression beider ABA-8-Hydroxylase-Gene, ABA8ox1 und

ABA8ox2, in kältetoleranten Genotypen höher (Nambara und Marion-Poll 2005; Oliver et al. 2007). Diese in Reis beobachteten ABA-Regulationsmechanismen könnten auch auf Sorghum übertragbar sein.

Gibberelline sind eine Gruppe verschiedener Gibberellinsäuren, von denen inzwischen über 100 unterschiedliche Verbindungen beschrieben sind. In Bezug auf Kältetoleranz wurden in Reispflanzen bereits GA4 und GA7 als beteiligte Hormone identifiziert. Kältestress führte in empfindlichen Genotypen zu einer Verringerung des endogenen Spiegels bioaktiver GA. Kältetolerante Reis-Genotypen hingegen sind in der Lage, über veränderte Biosynthesewege von GA4 und GA7 einen ausreichend hohen Pool bioaktiver GA aufrechtzuerhalten (Sharma und Nayyar 2016). Diese Unterschiede zwischen kältetolerantem und kälteempfindlichem Genotyp lassen sich bei Sorghumhirse in der Studie von Neitzert et al. (2026) nicht bestätigen. Beide Genotypen wiesen ähnlich hohe GA-Spiegel nach der Kälteexposition auf. Allerdings zeigte sich in BBCH39 unter dem Einfluss von Kälte ein signifikant geringerer GA-Gehalt. Aufgrund des gleichen Reaktionsmusters beider Genotypen scheint die GA-Biosynthese durch Kälte jedoch bei Sorghum nicht der limitierende Faktor zu sein. Züchtungsstrategien, die auf die Stabilisierung von GA-Pools abzielen, scheinen daher in Sorghum weniger angebracht als in Reis. Auch die Zusammensetzung des Pools an bioaktiven Gibberellinen scheint bei Sorghum von dem von Reis abzuweichen. Sowohl GA4 als auch GA7 lagen in Sorghum sowohl unter Stress als auch unter optimalen Bedingungen unterhalb der Nachweisgrenze.

Während die bestehende Literatur erhöhte ABA-Gehalte in Verbindung mit einem unzureichenden Pool bioaktiver Gibberelline meist als Folge einer gestörten Pollenentwicklung bei empfindlichen Pflanzen interpretiert (Sharma und Nayyar 2016), zeigt diese Arbeit, dass dies für Sorghum nicht zutrifft:

(i) Die hohen ABA-Gehalte im empfindlichen Genotyp gehen nicht mit einer verminderten Pollenfertilität einher (siehe Kapitel 4.2);

(ii) Die GA-Pools von tolerantem und empfindlichem Genotyp sind auf einem Level;

(iii) Der geringere GA-Gehalt nach Kälteexposition in BBCH39 beider Genotypen geht nicht mit geringerer Pollenfertilität einher.

Dies stützt die Annahme, dass die Pollenfertilität in Sorghum durch Kälte weniger eingeschränkt wird und eher eine Sensitivität des weiblichen Reproduktionsorgans eine Rolle spielt (siehe Kapitel 4.2).

Darüber hinaus wurden das Phytohormon Jasmonsäure (JA) und ihre bioaktive Form Jasmonsäure-Isoleucin (JAIle) als Signalstoffe für Stress- und Abwehrreaktionen in mehreren Studien mit Kältestress in Verbindung gebracht. Cai et al. (2014) zeigen, dass JA eine wichtige Rolle bei der Entwicklung generativen Gewebes spielt. Weitere Studien zeigen, dass in Reis mehrere JA-Biosynthesegene (u. a. AOC, AOS1, AOS2, LOX2) sowie Signal-Gene (COI1a, bHLH148) positiv auf Kältestress reagieren (Du et al. 2013; Hu et al. 2017). Bei Sorghumhirse zeigen sich die JA-Level durch Kälteexposition unabhängig vom Genotyp (tolerant und empfindlich) nicht verändert (Neitzert et al. 2026). Im Vergleich zu anderen Gräsern deutet dies auf eine geringe JA-Akkumulation unter Kältestress hin (Cai et al. 2014; Du et al. 2013). Ohne erhöhte JA-Akkumulation scheint die Expression der genannten JA-Biosynthesegene bei niedrigen Temperaturen in Sorghum – anders als in Reis (Du et al. 2013) – nicht durch Kälte induziert zu werden. Zur Prüfung dieser Hypothese wären quantitative Genexpressionsanalysen (qPCR) oder Transkriptom-Analysen sinnvoll, um zu klären, ob JA-Biosynthesegene in Sorghum tatsächlich nicht kälteinduzierbar sind. Eine andere Möglichkeit wäre jedoch, dass der JA-Biosyntheseweg im kältetoleranten Genotyp nicht grundsätzlich inaktiv ist, sondern sich vom empfindlichen Genotyp in der Verzweigung des Stoffwechselwegs unterscheidet. Neben JAIle kann aus JA im Cytoplasma auch das Derivat Methyljasmonat (MeJA) gebildet werden. MeJA wurde bereits mit reduzierten Kälteschäden bei *Cucurbita pepo* L. in Verbindung gebracht (Wang und Buta 1994). Die zukünftige Untersuchung von MeJA, idealerweise gemeinsam mit Expressionsanalysen der JA-Biosynthesegene, könnte daher zusätzliche Einblicke in die Rolle von JA und seinen Derivaten in der Kältetoleranz von Sorghum liefern.

Verschiedene Studien haben gezeigt, dass GA und JA in mehreren Signalwegen antagonistisch oder synergetisch miteinander interagieren. Durch das Zusammenspiel der beiden Gegenspieler können Wachstum und Anpassung an die vorherrschenden Umweltbedingungen justiert werden. Die Interaktionen finden sowohl unter optimalen als auch unter Stressbedingungen statt (Achard et al. 2008; Um et al. 2018). Bei Sorghum konnte gezeigt werden, dass die bioaktive Form JAIle und GA antagonistisch interagieren. Während

die JAIle-Konzentration mit dem Alter der Pflanze ansteigt – unabhängig von Genotyp und Umweltbedingungen – nimmt die GA-Konzentration progressiv ab und hat ihren Höchststand zu Beginn der frühen generativen Phase (Neitzert et al. 2026). In welchem Ausmaß der JA-GA-Crosstalk Wachstum und Entwicklung unter Stress reguliert, muss in weiteren Untersuchungen geklärt werden.

5.4 Vererbung und Züchtung

Die Vererbung abiotischer Stresstoleranzen, einschließlich Kältetoleranz, ist sehr komplex (Thomashow 1999; Sanghera et al. 2011). Für die Ausweitung des Anbaus von Sorghum in die gemäßigten Regionen Europas ist die reproduktive Kältetoleranz ein wichtiges Zuchtziel. Um den Züchtungsprozess wirksam zu verbessern, ist es maßgeblich, die genetische Grundlage dieser Merkmale zu verstehen.

Für die kommerzielle Nutzung wird Sorghumhirse überwiegend als F1-Hybride vermarktet. Eine leistungsfähige Hybridzüchtung setzt den Einsatz unterschiedlicher heterotischer Pools voraus, die sich durch eine möglichst große genetische Distanz auszeichnen. Hierdurch kann die maximale Heterosis ausgeschöpft und die allgemeine Kombinationsfähigkeit (GCA) verbessert werden. Die spezifische Kombinationsfähigkeit (SCA) spielt hier eine untergeordnete Rolle (Reif et al. 2005; Schnable und Springer 2013). Bisher gibt es bei Sorghum, im Gegensatz zu Mais, keine klar definierten heterotischen Pools (Jordan et al. 2003, Monk et al. 2014). Bei der Schaffung heterotischer Pools ist es sinnvoll, bei maternaler oder paternaler Vererbung die Eigenschaft gezielt in einem der Pools zu fokussieren. Bei biparental vererbten Merkmalen kann in beiden Pools selektiert werden (Becker 2019). In der vorliegenden Studie von Neitzert et al. (2025) konnte gezeigt werden, dass in Bezug auf reproduktive Kältetoleranz keine Unterschiede zwischen den beiden reziproken Hybriden vorliegen. Dies weist auf das Fehlen überwiegender mütterlicher oder väterlicher Vererbungseffekte hin. Aus Sicht der Hybridzüchtung gibt es daher keinen zwingenden Grund, Kältetoleranz vorrangig in nur einem der beiden Pools zu selektieren. Dies deckt sich auch mit anderen komplexen, kerngenetischen Merkmalen wie Trockentoleranz oder Kornertrag bei Sorghumhirse, welche ebenfalls biparental vererbt werden (Kebede et al. 2001; Boyles et al. 2017).

Des Weiteren zeigte Schaffasz et al. (2019b), dass reproduktive Kältetoleranz hinsichtlich der Merkmale Korngewicht, Kornanzahl und PHI einen heterotischen Charakter aufweist. Für das Merkmal PHI wurden ausgeprägte Effekte der GCA beobachtet. Zudem zeigte sich in vielen Kombinationen eine deutliche Mid-Parent-Heterosis, während Better-Parent-Heterosis nur selten auftrat, was auf eine überwiegend dominante Vererbung dieses Merkmals hinweist. Im Gegensatz zu diesen Befunden zeigt die in der von Neitzert et al. (2025) untersuchte Hybridkombination keine ausgeprägte Heterosis, sondern eine überwiegend additive Vererbung. Die additive Vererbung von QTL, welche zur Kältetoleranz in der reproduktiven Entwicklung beitragen, wurde bereits bei Reis beschrieben (Suh et al. 2010). Ein Argument zugunsten einer vermuteten heterotischen, dominanten Vererbung (F1 entspricht dem besseren Elternteil) lässt sich in dieser Hybridkombination lediglich für Kältestress ab BBCH32 formulieren. Es ist jedoch zu berücksichtigen, dass diese Studie nur eine Hybridkombination betrachtete – im Gegensatz zu den 49 F1-Hybridkombinationen bei Schaffasz et al. (2019b). Es ist allgemein anerkannt, dass die Ausprägung der Heterosis kombinationsspezifisch ist und tendenziell mit größerer genetischer Distanz der Eltern zunimmt. Beide Inzuchtlinien, SB14011 und SC1056, stammen aus dem Restorer-Pool des JLU-Sorghum-Züchtungsprogramms. Dies könnte die Heterosis in der resultierenden Intra-Pool-Kreuzung begrenzt haben – im Unterschied zu Schaffasz et al. (2019b), wo Inter-Pool-Kreuzungen geprüft wurden. Die Ergebnisse hinsichtlich des Ausbleibens einer High-Parent-Heterosis sind in beiden Studien jedoch übereinstimmend.

Während Sorghum vor Jahrtausenden noch durch einfache Handauslese auf gewünschte Merkmale hin „gezüchtet“ wurde (Dillon et al. 2007), steht heute ein breites Spektrum moderner Werkzeuge zur Verfügung, die den Züchtungsprozess effizienter gestalten. Im Kontext zur reproduktiven Kältetoleranz von Sorghum, welche als komplexes Merkmal der Beteiligung vieler Gene unterliegt, bedeutet das für die Züchtung:

(i) präzise, genetische, hochaufgelöste Erfassung der zugrundeliegenden Variation **(ii)** stabilere Phänotypisierung in den gegenüber Kälte empfindlichen Entwicklungsstadien **(iii**) bessere Vorhersage über Umwelten hinweg

Zur Verknüpfung von Genotyp- und Phänotypdaten stellen für komplexe Merkmale wie reproduktive Kältetoleranz beispielsweise Next-Generation-Sequencing (NGS), „Nested-Association Mapping“-Populationen (NAM) (Marla et al. 2019) oder „back-cross-NAM“

(BCNAM) einen zentralen Hebel dar (Jordan et al. 2011). NGS-basierte Genotypisierung (z. B. RAD-seq, GBS) macht es möglich, tausende Marker für Kartierung und Selektionsentscheidungen nutzbar zu machen (Nelson et al. 2011; Morris et al. 2013). Für Rückkreuzprogramme erlaubt dies nicht nur die Hintergrundselektion auf Elite-Genetik, sondern auch die genomische Selektion (GS), bei der der Zuchtwert von Individuen anhand genomweiter Markerinformation geschätzt wird. Für die Züchtung auf abiotische Stresstoleranzen wie Hitze- und Trockenstress hat sich die GS in anderen Kulturen bereits als geeignet erwiesen (Bhandari et al. 2019; Yuan et al. 2019; Trachsel et al. 2019). Auch bei Sorghumhirse wurde die GS u. a. für Wuchshöhe, Biomasse, Kornertrag und Stay-Green erfolgreich eingesetzt (Watanabe et al. 2017; Fernandes et al. 2018; Velazco et al. 2019). Für die hier diskutierte reproduktive Kältetoleranz ist GS besonders attraktiv, weil die phänotypische Erfassung arbeitsintensiv ist und stark von Umwelt- und Entwicklungsstadium abhängt.

Auch in der Phänotypisierung wurden durch Hochdurchsatzverfahren Fortschritte erzielt. Unbemannte Systeme (UAV) und sensorbasierte Plattformen ermöglichen die objektive Erfassung agronomischer Merkmale (z. B. Reife), während KI-basierte Bildanalyse komplexere Merkmale wie Rispenanzahl automatisiert erfassen kann (Watanabe et al. 2017; Chapman et al. 2018; Ghosal et al. 2019). Für reproduktive Kältetoleranz reicht jedoch „klassisches“ Feld-High-Throughput allein nicht aus, da die entscheidenden Prozesse in einem engen Zeitfenster stattfinden (Schaffasz et al. 2019a; Neitzert et al. 2025). Daraus ergibt sich ein praktischer Zuchtansatz: zweistufige Selektion, bei der zunächst über Feld-/UAV-Phänotypen eine Vorselektion erfolgt und anschließend eine gezielte, entwicklungsstadiumsgenaue Stressprüfung (Klimakammer-Versuche). Im Kontext der gezielten Phänotypisierung sollte zukünftig das weibliche Blütenorgan in Screening-Protokolle integriert werden, um die genaue Ursache für den geringen Kornertrag zu identifizieren und Kandidatengene zu finden. Dabei sollte der Fokus auf der bereits diskutierten eingeschränkten Rezeptivität des Pistils liegen (Osuna-Ortega et al. 2003; Thakur et al. 2010). Wenn das weibliche Blütenorgan den Engpass darstellt, rückt die Frage in den Vordergrund, welche Signale diese Einschränkung unter Kältestress im reproduktiven Stadium auslösen. Die gewonnenen Erkenntnisse auf hormoneller Ebene deuten darauf hin, dass die Fähigkeit, ABA-Spiegel unter Kälte zu begrenzen, ein zentrales Unterscheidungsmerkmal zwischen tolerantem und empfindlichem Genotyp ist. Das Aufrechterhalten niedriger ABA-Spiegel unter Kältestress im toleranten

Genotyp liefert einen plausiblen physiologischen Marker, der in genetische Modelle übersetzbar ist. Für die Züchtung heißt das, dass Kandidatenwege wie die ABA-Homeostase priorisiert werden sollten. Gleichzeitig eignen sich weitere Metabolom- und Phytohormonmessungen als Validierungsmarker in GS-Trainingspopulationen. Ressourcen wie QTL-Atlanten erleichtern zudem die Kandidatengen-Analyse und die Priorisierung von Regionen für experimentelle Validierung (Mace et al. 2019). Sobald robuste Kandidatengene vorliegen, eröffnen präzise Genomeditierungsverfahren die Möglichkeit, kausale Varianten in Elitehintergründen direkt zu testen oder günstige Allele gezielt zu erzeugen – insbesondere bei Signalwegen, in denen bereits aus anderen Arten funktionelle Anker bekannt sind (z. B. ABA-Homeostase-Gene) (Nambara & Marion-Poll 2005; Wolter et al. 2019). Umgekehrt können ungünstige Allele ebenfalls durch Gene-Editing ausgeschaltet werden („Knockout“).

6 Zusammenfassung

Die aus den semiariden Regionen Afrikas stammende Sorghumhirse birgt ein hohes Potential für die Ausweitung des Anbaus in die gemäßigten Breiten: eine hohe Wasser- und Nährstoffnutzungseffizienz, vielseitige Nutzungsmöglichkeiten, Resistenz gegen den Maiswurzelbohrer (*Diabrotica virgifera virgifera*) und ein mit Mais vergleichbares Ertragspotenzial mit deutlichen Vorteilen unter Trockenstress. Für eine Etablierung in den gemäßigten Regionen Europas, wie Deutschland, stellt die Kälteempfindlichkeit im reproduktiven Stadium eine zentrale Hürde dar. Daraus ergibt sich ein klarer Forschungs- und Züchtungsbedarf, der ein physiologisches Verständnis der zugrunde liegenden Mechanismen voraussetzt.

Die frühe reproduktive Phase ist bei Sorghumhirse das empfindlichste Zeitfenster gegenüber Kälte. Eine Akklimatisation nach Kältestress vor BBCH39 konnte nicht beobachtet werden. Es konnte gezeigt werden, dass Kälteexposition in frühen Stadien nicht nur zu einem reduzierten Kornansatz, sondern auch zu einer verringerten Rispenlänge führen kann – damit entstehen Ertragsverluste potenziell über zwei Wege: weniger Befruchtung (d. h. reduzierter Kornansatz) und weniger Blütchen (d. h. unabhängig vom Befruchtungserfolg bereits reduzierte Kornanzahl durch kürzere Rispen). Ein zentrales Ergebnis ist zudem die Relativierung der in der Literatur beschriebenen, durch Kälte eingeschränkten Pollenfertilität mit einhergehenden Ertragsverlusten. Entgegen früherer Annahmen gingen bei den untersuchten Genotypen Ertragsminderungen nicht zwingend mit verringerter Pollenfertilität einher. Zusätzliche Bestäubungsexperimente zeigten, dass die männliche Komponente nicht der erstlimitierende Faktor ist. Diese Ergebnisse sprechen dafür, dass Kälte den Ertrag nicht primär über eine eingeschränkte Pollenfertilität begrenzt – sondern dass das weibliche Blütenorgan vermutlich stärker als bisher angenommen zur kälteinduzierten Ertragsdepression beitragen könnte.

Auf hormoneller Ebene konnte außerdem gezeigt werden, dass der kältetolerante Genotyp in der Lage ist, Abscisinsäure unter Kältestress niedrig zu halten, während der empfindliche Genotyp höhere Konzentrationen aufweist. Im Unterschied zu Reis lässt sich der dort postulierte Mechanismus, das hohe Abscisinsäure-Gehalte und ein zu geringer Pool an bioaktiven Gibberellinen zu einer gestörten Pollenentwicklung führen, für Sorghum nicht bestätigen: (i) hohe Abscisinsäure-Spiegel korrelieren hier nicht zwingend mit reduzierter Pollenfertilität, (ii) Gibberellinsäure-Pools sind zwischen tolerant und empfindlich weitgehend

ähnlich, und (iii) ein kältebedingter Rückgang an Gibberellinen in BBCH39 beider Genotypen stellt keine Erklärung für die Pollenreaktion dar. Zusätzlich wurde ein antagonistischer Zusammenhang zwischen Gibberellinsäure und Jasmonsäure unabhängig von Umweltbedingungen und Genotyp beobachtet. Eine klare kälteinduzierte Akkumulation von Jasmonsäure, wie in anderen Gräsern berichtet, zeigte sich in Sorghum nicht, was alternative Pfade (z. B. MeJA) oder artspezifische Regulation nahelegt.

Reproduktive Kältetoleranz ist ein komplexes, polygenes Merkmal. Hinsichtlich der Vererbung zeigen F1-Hybriden keine überwiegenden maternalen oder paternalen Effekte, was für eine biparentale Vererbung spricht und somit gegen die Möglichkeit, Toleranz ausschließlich in einem heterotischen Pool zu fixieren. Gleichzeitig zeigt der Vergleich mit früheren Arbeiten, dass Heterosis-Effekte stark kombinationsabhängig sind und die hier beobachtete Vererbung eher additiv erscheint. Für die Züchtung sollten Screening-Protokolle um weibliche Reproduktionsmerkmale ergänzt werden. Als zentraler physiologischer Marker bzw. Kandidatenpfad bietet sich die ABA-Homeostase an. Ergänzend können NGS-Genotypisierung, NAM/BCNAM und vor allem genomische Selektion helfen, Leistungen stabil über verschiedene Umwelten hinweg vorherzusagen – gestützt durch Hormon-/Metabolommarker und später durch funktionelle Validierung bis hin zu gezielter Genomeditierung, sobald belastbare Kandidatengene vorliegen. Aufgrund der begrenzten Anzahl an Genotypen sollten zukünftige Studien eine breitere genetische Diversität einbeziehen, um die Rolle der weiblichen Organe und die genetischen Determinanten der Kältetoleranz präziser zu klären.

Zusammenfassend leistet diese Arbeit einen Beitrag zum besseren Verständnis der reproduktiven Kältetoleranz in *Sorghum bicolor*. Auf physiologischer und genetischer Ebene schärfen die Ergebnisse das Verständnis der zugrundeliegenden Mechanismen. Des Weiteren eröffnen sie Perspektiven für künftige Forschungs- und Züchtungsarbeiten hinsichtlich kältetoleranter und ertragsstabiler Sorghumsorten für die Ausweitung des Anbaus in gemäßigte Breiten, insbesondere vor dem Hintergrund globaler Ernährungs- und Ressourcenherausforderungen.

7 Summary

Sorghum, originating from semi-arid regions, has high potential for expanding cultivation into temperate zones due to its high water and nutrient use efficiency, versatile uses, resistance to the western corn rootworm (*Diabrotica virgifera virgifera*), and a yield potential similar to maize – while requiring substantially less water. For broader establishment in temperate regions such as Germany, cold sensitivity during the reproductive stage remains a major barrier. This creates a clear need for research and breeding that is grounded in a physiological understanding of the underlying mechanisms.

The early reproductive phase is the most cold-sensitive time window in sorghum. No acclimation after cold stress prior to BBCH39 was observed. Cold exposure at early stages can reduce not only seed set but also panicle length, inducing yield losses via two pathways: less fecundation (i. e. reduced seed set) and less florets (i. e. less potential seeds regardless of fecundation success). A key outcome is the re-evaluation of the literature's common assumption that cold-induced reductions in pollen fertility directly drive yield losses. Contrary to earlier assumptions, yield reductions in the genotypes studied were not necessarily accompanied by decreased pollen fertility. Additional pollination experiments indicated that the male component is not the most limiting factor. These results suggest that cold does not primarily limit yield via reduced pollen fertility, but that the female floral organs may contribute more strongly than previously assumed to cold-induced yield depression.

A closer look at the hormonal level showed that the cold-tolerant genotype is able to keep abscisic acid (ABA) low under cold stress, whereas the sensitive genotype shows higher concentrations. In contrast to rice, the proposed mechanism that high ABA levels together with an insufficient pool of bioactive gibberellins lead to impaired pollen development cannot be confirmed for sorghum: (i) high ABA levels do not necessarily correlate with reduced pollen fertility, (ii) gibberellin pools are largely similar between tolerant and sensitive genotypes, and (iii) a cold-induced decrease in gibberellins at BBCH39 in both genotypes does not explain the pollen response. In addition, an antagonistic relationship between gibberellins and jasmonic acid was observed independently of genotype and environmental conditions. A clear cold-induced accumulation of jasmonic acid, as reported for other grasses, was not detected in sorghum, suggesting alternative routes (e.g., MeJA) or species-specific regulation.

Reproductive cold tolerance is a complex, polygenic trait. Regarding inheritance, F1 hybrids showed no predominant maternal or paternal effects, supporting mainly biparental inheritance and arguing against the possibility to fix tolerance exclusively in a single heterotic pool. At the same time, comparison with earlier work indicates that heterosis effects are highly combination-dependent, and the inheritance observed here appears more additive. For breeding, screening protocols should be expanded to include female reproductive traits. ABA homeostasis represents a central physiological marker and candidate pathway. In addition, NGS-based genotyping, NAM/BCNAM, and especially genomic selection can help predict performance more robustly across environments – supported by hormone/metabolome markers and later functional validation up to targeted genome editing once robust candidate genes are available. Given the limited number of genotypes, future studies should include broader genetic diversity to clarify the role of female organs and the genetic determinants of cold tolerance more precisely.

In summary, this work contributes to a better understanding of reproductive cold tolerance in *Sorghum bicolor*. At the physiological and genetic levels, the results refine our understanding of the underlying mechanisms. They also provide perspectives for future research and breeding efforts towards cold-tolerant and yield-stable sorghum cultivars to support the expansion of cultivation into temperate regions, particularly in light of global food and resource challenges.

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9 Eidesstattliche Erklärung

Erklärung gemäß § 17 (2) der Promotionsordnung des Fachbereichs 09 vom 07.07.2004 in der Fassung des 2. Änderungsbeschlusses vom 19.05.2019

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Kreuztal 18.02.2026 **Luisa Neitzert**

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11 Anhang

Anhang I: Ergänzendes Material zu Publikation I

Neitzert, L., Kravcov, N., Wittkop, B., Snowdon, R., and Windpassinger, S. (2025). Reproductive cold stress in contrasting sorghum genotypes: is pollen fertility really the crucial trait? Plant Direct 9, e70065. doi: 10.1002/pld3.70065.

3.1 Sensitivity to ocld in the reproductive stage

Table S1: The result of the Least Significant Difference (LSD) post-hoc analysis to determine significant differences which treatments showed statistically significant differences from each other in terms of Panicle harvest index (PHI) and seed yield [g], cell concentration [cells/ml], total number of fertile pollen [cells/ml], proportion of fertile pollen [%]

SB14011	**PHI**					**seed yield [g]**					**cell concentration [cells/ml]**					**total number of fertile pollen [cells/ml]**					**proportion of fertile pollen [%]**				
ANOVA	p=0.159					p=0.211					p= 1.18e-13 ***					p= 1.47e-12 ***					p= 4.95e-07 ***				
contrasts	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**
BBCH32-BBCH35	/	/	/	/	/	/	/	/	/	/	-2172.1	354	155	-6.138	<.0001	-1935.6	311	155	-6.233	<.0001	-0.1181	0.0276	155	-4.279	<.0001
BBCH32-BBCH39	/	/	/	/	/	/	/	/	/	/	-1476.8	354	155	-4.173	<.0001	-1447.7	311	155	-4.662	<.0001	-0.1285	0.0276	155	-4.655	<.0001
BBCH32-BBCH51	/	/	/	/	/	/	/	/	/	/	350.5	340	155	1.031	0.3041	-10.7	298	155	-0.036	0.9714	-0.1484	0.0265	155	-5.597	<.0001
BBCH32-control	/	/	/	/	/	/	/	/	/	/	332.7	354	155	0.94	0.3485	145.4	311	155	0.468	0.6403	-0.0739	0.0276	155	-2.678	0.0082

BBCH 35-BBCH 39	/	/	/	/	/	/	/	/	/	/	695.4	367	155	1.894	0.0601	488	322	155	1.514	0.132	-0.0104	0.0286	155	-0.363	0.7173
BBCH 35-BBCH 51	/	/	/	/	/	/	/	/	/	/	2522.7	354	155	7.129	<.0001	1924.9	311	155	6.199	<.0001	-0.0303	0.0276	155	-1.098	0.2737
BBCH 35-control	/	/	/	/	/	/	/	/	/	/	2504.9	367	155	6.821	<.0001	2081	322	155	6.458	<.0001	0.0442	0.0286	155	1.543	0.1249
BBCH 39-BBCH 51	/	/	/	/	/	/	/	/	/	/	1827.3	354	155	5.164	<.0001	1437	311	155	4.628	<.0001	-0.0199	0.0276	155	-0.722	0.4713
BBCH 39-control	/	/	/	/	/	/	/	/	/	/	1809.5	367	155	4.928	<.0001	1593	322	155	4.944	<.0001	0.0546	0.0286	155	1.905	0.0586
BBCH 51-control	/	/	/	/	/	/	/	/	/	/	-17.8	354	155	-0.05	0.9599	156.1	311	155	0.503	0.6159	0.0745	0.0276	155	2.699	0.0077

SC1056	PHI					seed yield [g]					cell concentration [cells/ml]					total number of fertile pollen [cells/ml]					proportion of fertile pollen [%]				
ANOVA	p= 0.00841**					p=1.58e-05***					p= 5.42e-05 ***					p= 0.000219 ***					p= 5.64e-07 ***				
contrasts	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**
BBCH 32-BBCH 35	0.0669	0.136	10	0.491	0.634	0.527	2.31	10	0.228	0.824	1359.7	311	165	4.366	<.0001	1140.1	281	165	4.054	0.0001	0.14309	0.0377	165	3.798	0.0002
BBCH 32-BBCH 39	-0.0196	0.136	10	-0.144	0.8884	0.873	2.31	10	0.379	0.7129	650.6	341	165	1.907	0.0583	503.6	308	165	1.635	0.104	0.00324	0.0413	165	0.079	0.9375
BBCH 32-BBCH 51	-0.4104	0.136	10	-3.012	0.0131	-11.707	2.31	10	-5.075	0.0005	801.8	329	165	2.439	0.0158	464.8	297	165	1.566	0.1193	-0.06343	0.0398	165	-1.595	0.1126
BBCH 32-control	-0.4337	0.136	10	-3.184	0.0098	-18.58	2.31	10	-8.054	<.0001	62.3	341	165	0.183	0.8553	16.4	308	165	0.053	0.9576	-0.01285	0.0413	165	-0.311	0.756
BBCH 35-BBCH 39	-0.0865	0.136	10	-0.635	0.5396	0.347	2.31	10	0.15	0.8835	-709.1	311	165	-2.277	0.0241	-636.5	281	165	-2.263	0.0249	-0.13985	0.0377	165	-3.712	0.0003
BBCH 35-BBCH 51	-0.4773	0.136	10	-3.504	0.0057	-12.233	2.31	10	-5.303	0.0003	-558	298	165	-1.874	0.0628	-675.3	269	165	-2.511	0.013	-0.20652	0.036	165	-5.733	<.0001

BBCH 35-control	-0.5006	0.136	10	-3.675	0.0043	-19.107	2.31	10	-8.283	<.0001	-1297.4	311	165	-4.166	<.0001	-1123.7	281	165	-3.996	0.0001	-0.15594	0.0377	165	-4.14	0.0001
BBCH 39-BBCH 51	-0.3907	0.136	10	-2.868	0.0167	-12.58	2.31	10	-5.453	0.0003	151.1	329	165	0.46	0.6464	-38.8	297	165	-0.131	0.8961	-0.06667	0.0398	165	-1.677	0.0955
BBCH 39-control	-0.4141	0.136	10	-3.04	0.0125	-19.453	2.31	10	-8.433	<.0001	-588.3	341	165	-1.724	0.0865	-487.2	308	165	-1.581	0.1157	-0.01609	0.0413	165	-0.39	0.6972
BBCH 51-control	-0.0233	0.136	10	-0.171	0.8674	-6.873	2.31	10	-2.98	0.0138	-739.5	329	165	-2.249	0.0258	-448.4	297	165	-1.51	0.1329	0.05058	0.0398	165	1.272	0.2052

SBxSC	**PHI**					**seed yield [g]**					**cell concentration [cells/ml]**					**total number of fertile pollen [cells/ml]**					**proportion of fertile pollen [%]**				
ANOVA	p= 0.357					p=0.29					p= 3.69e-10 ***					p= 1.39e-07 ***					p= 0.00482 **				
contrasts	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**
BBCH 32-BBCH 35	/	/	/	/	/	/	/	/	/	/	616	313	160	1.969	0.0507	643.8	289	160	2.231	0.0271	0.0536	0.0269	160	1.991	0.0482
BBCH 32-BBCH 39	/	/	/	/	/	/	/	/	/	/	1111	320	160	3.472	0.0007	1041.3	295	160	3.53	0.0005	0.0669	0.0275	160	2.43	0.0162

BBCH 32-BBCH 51	/	/	/	/	/	/	/	/	/	/	2175	302	160	7.206	<.0001	1734.8	278	160	6.236	<.0001	-0.0188	0.026	160	-0.722	0.4712
BBCH 32-control	/	/	/	/	/	/	/	/	/	/	1225	323	160	3.797	0.0002	1092.1	297	160	3.672	0.0003	0.0338	0.0278	160	1.217	0.2255
BBCH 35-BBCH 39	/	/	/	/	/	/	/	/	/	/	495	310	160	1.595	0.1127	397.6	286	160	1.39	0.1665	0.0133	0.0267	160	0.498	0.6194
BBCH 35-BBCH 51	/	/	/	/	/	/	/	/	/	/	1558	291	160	5.347	<.0001	1091	269	160	4.06	0.0001	-0.0724	0.0251	160	-2.886	0.0044
BBCH 35-control	/	/	/	/	/	/	/	/	/	/	609	313	160	1.945	0.0536	448.4	289	160	1.554	0.1222	-0.0198	0.0269	160	-0.737	0.4625
BBCH 39-BBCH 51	/	/	/	/	/	/	/	/	/	/	1063	299	160	3.557	0.0005	693.4	276	160	2.516	0.0129	-0.0857	0.0257	160	-3.329	0.0011
BBCH 39-control	/	/	/	/	/	/	/	/	/	/	114	320	160	0.356	0.7226	50.8	295	160	0.172	0.8635	-0.0331	0.0275	160	-1.203	0.2307
BBCH 51-control	/	/	/	/	/	/	/	/	/	/	-950	302	160	-3.147	0.002	-642.6	278	160	-2.31	0.0222	0.0525	0.026	160	2.023	0.0447

SCxSB	PHI					seed yield [g]					cell concentration [cells/ml]					total number of fertile pollen [cells/ml]					proportion of fertile pollen [%]				
ANOVA	p= 0.224					p= 0.00448 **					p= 0.000989 ***					p= 0.000662 ***					p= 0.0775				
contrasts	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**	**estimate**	**SE**	**df**	**t.value**	**p.value**
BBCH 32-BBCH 35	/	/	/	/	/	3.01	5.06	10	0.595	0.565	1145.7	388	147	2.952	0.0037	1148.6	347	147	3.314	0.0012	/	/	/	/	/
BBCH 32-BBCH 39	/	/	/	/	/	-0.06	5.06	10	-0.012	0.9908	1085.9	370	147	2.937	0.0039	1018.8	330	147	3.085	0.0024	/	/	/	/	/
BBCH 32-BBCH 51	/	/	/	/	/	-19.31	5.06	10	-3.814	0.0034	1552.7	381	147	4.075	0.0001	1403.7	340	147	4.126	0.0001	/	/	/	/	/
BBCH 32-control	/	/	/	/	/	-13.65	5.06	10	-2.696	0.0225	1488.4	388	147	3.835	0.0002	1374.9	347	147	3.967	0.0001	/	/	/	/	/
BBCH 35-BBCH 39	/	/	/	/	/	-3.07	5.06	10	-0.607	0.5574	-59.8	326	147	-0.183	0.855	-129.8	292	147	-0.445	0.6569	/	/	/	/	/
BBCH 35-BBCH 51	/	/	/	/	/	-22.33	5.06	10	-4.409	0.0013	407.1	339	147	1.2	0.232	255.1	303	147	0.842	0.4009	/	/	/	/	/

BBCH 35-control	/	/	/	/	/	-16.67	5.06	10	-3.291	0.00081	342.7	347	147	0.987	0.3251	226.4	310	147	0.73	0.4664	/	/	/	/	/
BBCH 39-BBCH 51	/	/	/	/	/	-19.25	5.06	10	-3.802	0.00035	466.8	318	147	1.468	0.1442	384.9	284	147	1.355	0.1774	/	/	/	/	/
BBCH 39-control	/	/	/	/	/	-13.59	5.06	10	-2.684	0.0229	402.5	326	147	1.233	0.2196	356.1	292	147	1.222	0.2238	/	/	/	/	/
BBCH 51-control	/	/	/	/	/	5.66	5.06	10	1.118	0.2898	-64.3	339	147	-0.19	0.8498	-28.8	303	147	-0.095	0.9245	/	/	/	/	/

3.4 Parent lines vs. reciprocal F1 hybrids

Table S2: The result of the paired t-test to analyse significant differences between the parental lines SB14011 and SC1056 and their reciprocal F1 hybrids within a given treatment level in terms of Panicle harvest index (PHI) and seed yield [g]

treatment	**BBCH32**		**BBCH35**		**BBCH39**		**BBCH51**		**control**	
	PHI	**seed yield**	**PHI**	**seed yield**	**PHI**	**seed yield**	**PHI**	**seed yield**	**PHI**	**seed yield**
contrasts	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**	**p-value**
SB14011-SC1056	0.245	0.238	0.099	0.0094**	0.016*	0.011*	0.0232*	0.1521	0.0023	0.37
SB14011-SCxSB	0.437	0.702	0.475	0.0747	0.797	0.088	0.0085**	0.0972	0.0672	0.99
SB14011-SBxSC	0.43	0.206	0.384	0.0983	0.233	0.123	0.0119*	0.9867	0.0916	0.84
SC1056-SCxSB	0.072	0.133	0.298	0.2154	0.024*	0.206	0.5247	0.0086**	0.052	0.36
SC1056-SBxSC	0.07	0.029*	0.374	0.1662	0.116	0.149	0.6707	0.156	0.038*	0.29
SCxSB-SBxSC	0.989	0.355	0.867	0.8633	0.335	0.832	0.8285	0.0946	0.8464	0.86

3.4 Parent lines vs. reciprocal F1 hybrids

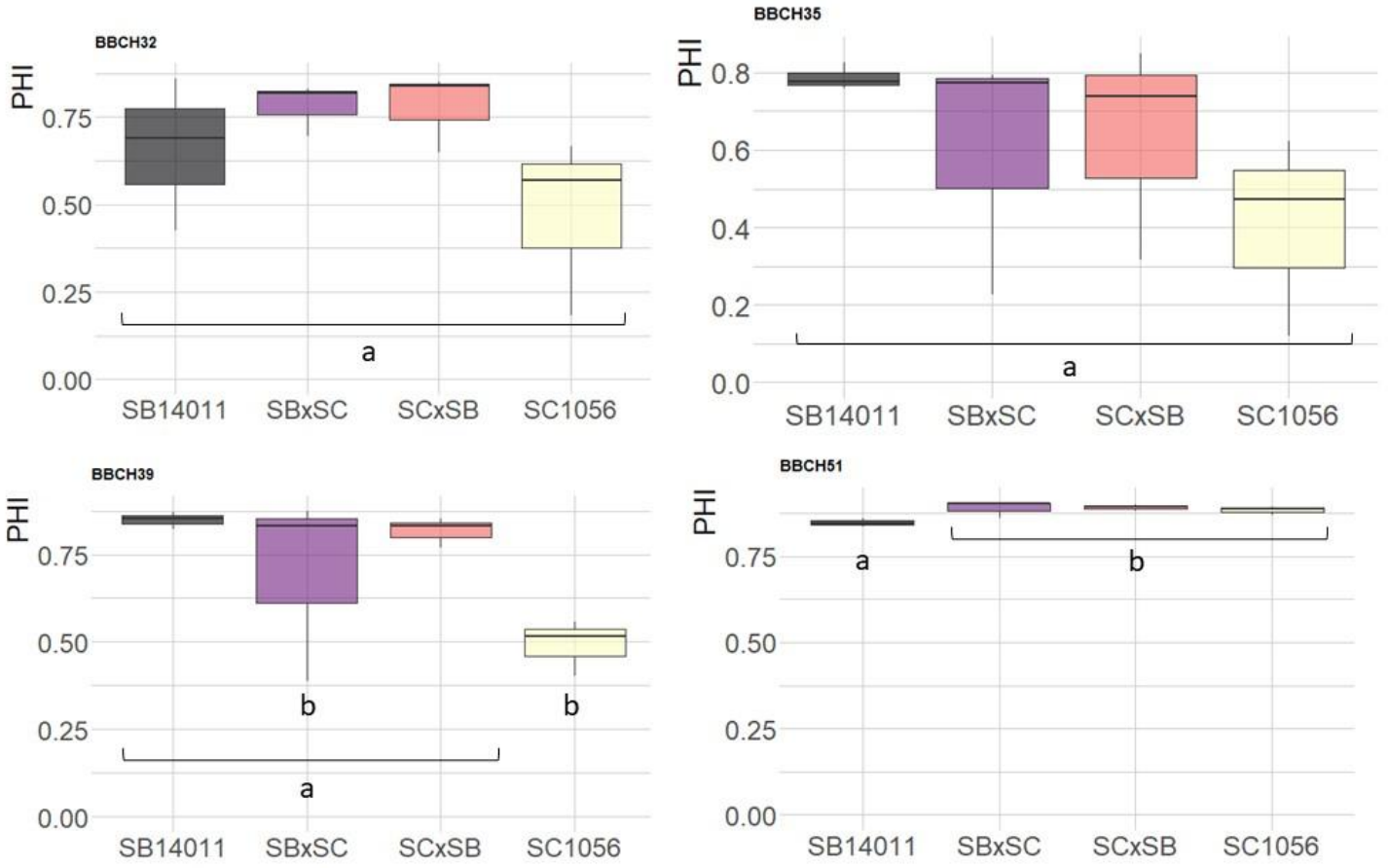

Figure 11a: Boxplots showing the Panicle harvest index of the cold-tolerant parental line SB14011 and the cold-sensitive parental line SC1056 and their reciprocal F1 hybrids in the different treatment stages (cold stress after BBCH32, BBCH35, BBCH39 and BBCH51); p-values in the supplementary part (Table y

Anhang II: Ergänzendes Material zu Publikation II

Neitzert, L., Kravcov, N., Tandron Moya, Y. A., Windpassinger, S., von Wirén, N., Snowdon, R., et al. (2026). Cold shock for cold tolerance: phytohormone dynamics in sorghum provides insights. Plant Direct 10, e70133. doi: 10.1002/pld3.70133.

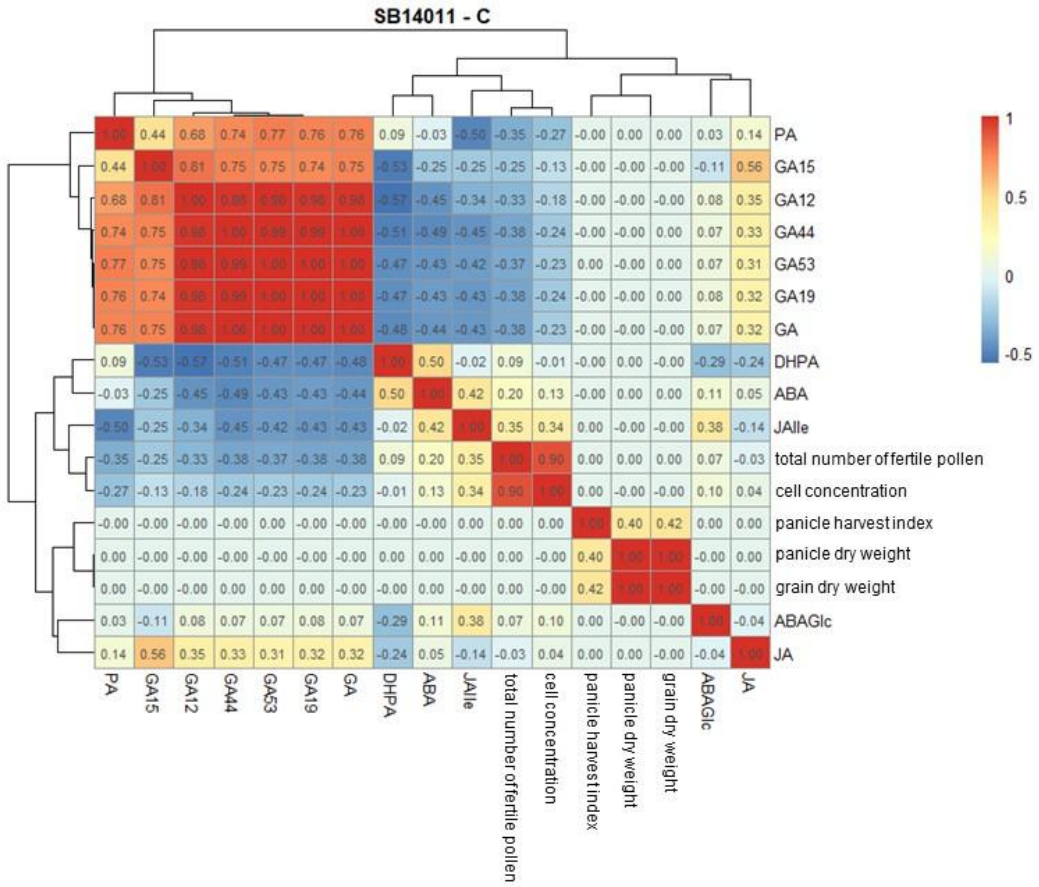

Figure 13: Heatmap illustrating Pearson's correlations among the analyzed traits for the cold-tolerant genotype SB14011 under optimal conditions: Abscisic acid (ABA), Abscisic acid glucose ester (ABAGe), Dihydrophaseic acid (DHPA), Jasmonic acid (JA), Phaseic acid (PA), gibberellins (GA) and their derivatives (GA12, GA15, GA19, GA24, GA44, and GA53), total number of fertile pollen, cell concentration, panicle harvest index, panicle dry weight, and grain dry weight. Strong positive correlations (r = 1) are shown in red, while strong negative correlations (r = -1) are shown in blue.

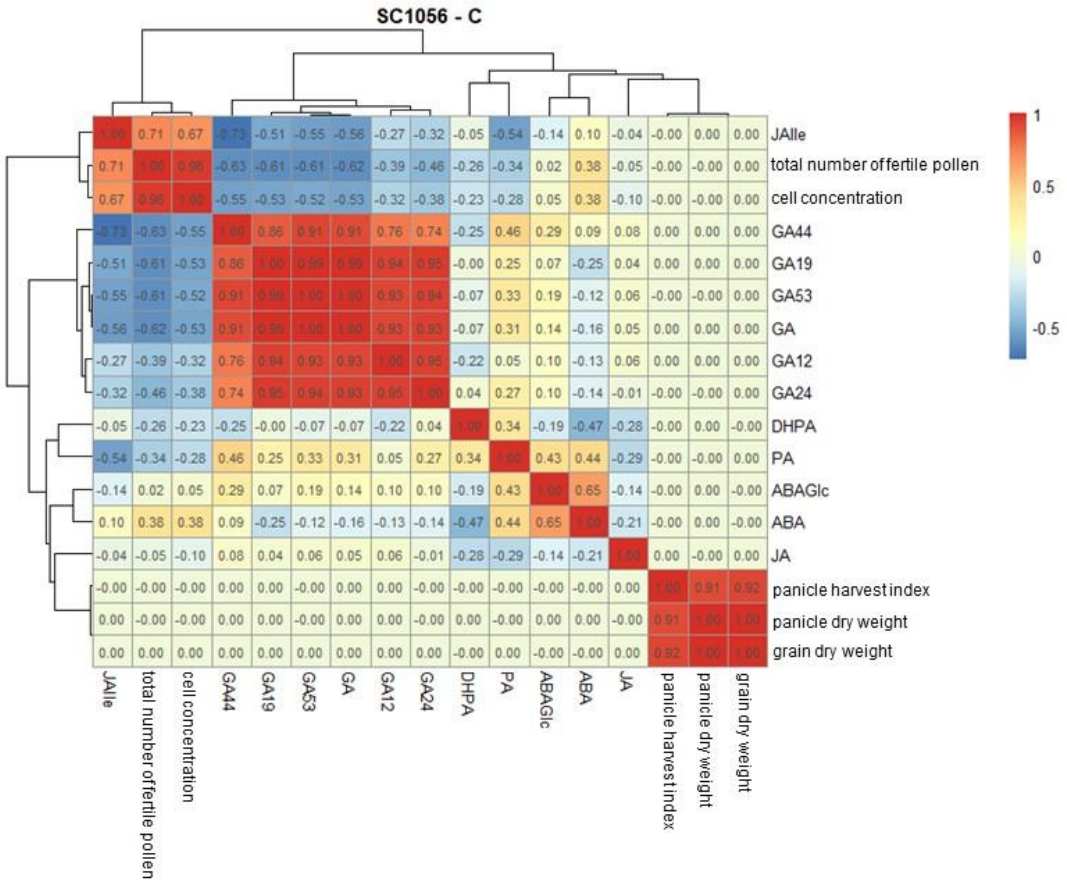

Figure 14: Heatmap illustrating Pearson's correlations among the analyzed traits for the cold-sensitive genotype SC1056 under optimal conditions: Abscisic acid (ABA), Abscisic acid glucose ester (ABAGe), Dihydrophaseic acid (DHPA), Jasmonic acid (JA), Phaseic acid (PA), gibberellins (GA) and their derivatives (GA12, GA15, GA19, GA24, GA44, and GA53), total number of fertile pollen, cell concentration, panicle harvest index, panicle dry weight, and grain dry weight. Strong positive correlations (r = 1) are shown in red, while strong negative correlations (r = -1) are shown in blue.