Ansøgning om brug af genomredigeret byg i sædskifteforsøg

Indhold

A. Generelle oplysninger

A.1 Anmelderens navn og adresse

Kim Hebelstrup, Aarhus Universitet, Forsøgsvej 1, 4200 Slagelse

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Anders Vestergaard, Aarhus Universitet, Forsøgsvej 1, 4200 Slagelse

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A.2 De ansvarligere forskeres navne

Kim Hebelstrup, Lektor, Ph.d., gruppeleder, Institut for Agroøkologi, Aarhus Universitet, Flakkebjerg, > 25 års erfaring i forskning, udvikling, analyse og håndtering af transgene og GMO klassificerede planter (Arabidopsis, byg, hvede og kartofler).

Markpersonalet har ved kursus på Bygholm Landbrugssskole (d. 5-6 februar) opnået GMO – kørekort. Anders Vestergaard er ansvarlig for, at kun personale med GMO-kørekort inddrages i forsøg.

Jordbrugsteknologer/markforsøgs personale fra Flakkebjerg der har GMO kørekort:

Hans Hansen
Thor Hougaard
Josefine Frost
Henrik Jespersen
Henrik Skydebjerg
Anders Vestergaard

Laborant der har GMO kørekort

Rikke Jacobsen (som har > 10 års erfaring med håndtering af GMO materiale, herunder GMO planter)

A.3 Projektets titel

Udsætning af genomredigeret byg i sædskifteforsøg

A.4 Udsætningen

A.4.A Formålet med udsætningen

Forsøgene vil foregå ved **Aarhus Universitet, Flakkebjerg**, Forsøgsvej 1, 4200 Slagelse (se desuden bilag over ansøgte områder)

Formålet er at teste CRISPR/Cas9 genom redigerede byg. For byg planterne ønskes det at undersøge muligheden for i sædskifte at producere byg med en betydelig forbedret foderkvalitet, opnået ved CRISPR/Cas9 induceret knock-out af protease inhibitoren chymotrypsin inhibitor 1A (herefter betegnet 'ci-1a')
Der bruges en blanding af lucerne og vikke i sædskiftet. Lucerne/vikke planterne i forsøget er ikke GMO.

A.4.B Udsætningens startdato og varighed.

Udsætning sker i perioden 15. marts – 15. juli og høst i perioden 01. september – 30. oktober i årene 2026-2028 i Flakkebjerg

A.4.C Udsætningsmetode

Byg og lucerne/vikke: Der udplantes fra frø i parceller

A.4.D Fremgangsmåde ved forberedelse og behandling af udsætningsstedet inden, under og efter udsætningen, herunder dyrknings- og høstpraksis:

Forår:

Marken er det første år pløjet og/eller harvet op inden udplantning af byg. De efterfølgende år vil der kun blive harvet.

Under (udsætningen) væksten:

Normal behandling mod ukrudt, skadedyr og sygdomsbekæmpelse. Byggen vækstreguleres efter behov og vil løbende blive gødet med kunstgødning (granulat eller flydende) og desuden vandet efter behov.

Høst:

Ved høst anvendes specialbygget forsøgsmejetærsker (se bilag), der er designet til at undgå sammenblanding af korn. Den er således designet til nemt at kunne rengøres for frø. Mejetærskeren rengøres ligeledes på anviste rengøringsplads (se bilag) på det ansøgte areal. Der vil altid være minimum 15 meter til øvrige dyrkede områder.

Høstede korn og andre dele fra byg planterne destrueres efter godkendte forskrifter for GMO plantemateriale eller fragtes efter godkendte forskrifter for GMO plantemateriale til GMO godkendte (klasse: planter) faciliteter (laboratorier og lokaler godkendt til opbevaring af GMO plantemateriale) som forefindes på matriklen i Flakkebjerg.

Al GMO materiale der analyseres i laboratorier destrueres ved laboratorieforsøgenes endelige afslutning. Således erklæres det udtrykkeligt, at intet af GMO materialet vil blive markedsført eller indgå i fødevare- eller foderkredsløbet.

Parceller med lucerne skæres og nedfræses i jordbunden. Lucerne/vikke dyrkes således kun med henblik på jordforbedring.

Alle maskiner der anvendes på det godkendte område rengøres ligeledes på anviste rengøringsplads (se bilag) på det ansøgte areal. Maskiner rengøres med 8 bar vandtryk og/eller trykluft som forefindes på stedet. Det sikres at spildevandet nedsiver på det anviste rengøringsområde.

A.4.E Omtrentlig antal planter per kvm.

Byg: 150 – 400 planter per kvm.

Lucerne/vikke: 50-100 planter per kvm (OBS: Disse planter er ikke GMO)

A.5 Oplysninger om udsætningsstedet

A.5.a Udsætningsstedets størrelse og beliggenhed

Udsætningsstedet er beliggende i markbloknummer 651133-27 Flakkebjerg.
Området, der vil blive tilplantet med afgrøderne, vil være 7.200 m² brutto og 720 m² netto.
Forskel mellem brutto og netto areal er værn og sti. Værn består af et slået græsareal og sort jord (se bilag).

A.5.b Beskrivelse af udsætningsstedets økosystem, herunder klima, flora og fauna.

Udsætningsstedet er beliggende i et konventionelt dansk landbrugsareal.

A.5.c Forekomsten af krydsningskompatible beslægtede vilde eller dyrkede plantearter.

Da byg er selvbestøvende er der ikke risiko for krydsning til beslægtede eller dyrkede plantearter. Der er altid minimum 15 meter til øvrige marker.

A.5.d Afstanden til officielt anerkendte biotoper eller beskyttede områder, som vil påvirkes (se bilag).

For arealet beliggende i markblok 651133-27 er afstanden:

§3 mose: 200 meter

Se desuden bilag 3 med oversigtskort.

B. Videnskabelige oplysninger

B.1 Oplysninger om recipientplanter eller - hvor det er relevant - forældreplanter

B.1.a Fuldstændige navne

Taxonomi	Latinske navn
i) Familie	*Poaceae*
ii) Slægt	*Hordeum*
iii) Art	*Hordeum vulgare*
iv) Underart	*vulgare*
v) Sort	Stairway
vi) Almindeligt navn	Byg

B.1.b Udbredelse og dyrkning i Unionen

Byg dyrkes bredt i alle lande i unionen og anvendes primært til fremstilling af dyrefoder og til fremstilling af maltkorn. Kun i meget begrænset omfang anvendes byg til fremstilling af øvrige fødevarer end malt.

Sorten Stairway er udmærket ved en lav modtagelighed for bygrust, bygbladplet og skoldplet samt højt udbyttepotentiale. Se desuden https://nordicseed.dk/saasaed/vaarsaed/vaarbyg/stairway for yderligere agronomisk karakterisering.

B.1.c Reproduktion

i)

Byg reproducerer ved frødannelse fra selvbestøvende blomster. Byg er diploid. Bygs haploide genom er på ca. 5,1 gigabasepar (Gb).

ii)

Byg er selvbestøvende, hvorfor risiko for krydsbestøvning anses som værende ubetydelig så længe afstandskrav overholdes.

iii)

Byg er 1. årig.

B.1.d Krydsningskompatibilitet med andre dyrkede eller vilde plantearter, herunder udbredelsen i Europa af de kompatible arter.

Der kendes ikke til krydsninger mellem byg og andre dyrkede eller vilde arter i Europa i marken. Krydsningskompatibiliteten må derfor anses for at være ikke eksisterende i marken. Det bemærkes igen, at der altid overholdes en afstand på mindst 15 meter til nærmeste øvrige marker.

B.1.e Overlevelsesevne:

i)

Evne til at danne strukturer, der fremmer overlevelse eller vækstdvale:

Byg er enårig. Der kan forekomme spildfrø.

Området overvåges månedligt efter høst for spildfrø af byg. Frø, og eventuelle planter spiret herfra fjernes straks manuelt. Ved sædskifte vil der altid mellem afgrøder blive bekæmpet med herbicid, så eventuelle spirede planter fra byg frø straks nedsprøjtes.

ii)

Ingen særlige faktorer.

B.1.f Spredning

i)

Der er ikke risiko for spredning af pollen, da byg er selvbestøvende.

ii)

Generel betragtning vedr. risiko for spredning

Da byggen hverken indeholder transgener eller cisgener er der ikke mulighed for spredning af transgener eller cisgener til mikroorganismer.

B.1.g

Ikke relevant

B.1.h

Byg vekselvirker ikke med andre planter eller organismer, hvor den dyrkes konventionelt, og der er ikke nogen kendt toksisk virkning på dyr eller andre organismer. Hordein fra frøhviden i enhver bygplante kan give en allergisk reaktion hos nogle mennesker med sygdommen cøliaki. Den her anvendte byg plante har en bedre evne til at stimulere nedbrydning af proteiner med proteaser. Det er derfor sandsynligt, at mængden af hordein vil være reduceret i produkter fra den her anvendte byg. Det understreges igen, at byg planten ikke har fået tilført fremmede trans-gene faktorer, og således ikke kan have en allergenicitet som adskiller sig fra den allergenicitet der allerede findes i byg.

B.2 Molekylær karakterisering

a) Oplysninger om den genetiske modifikation

Ved CRISPR/Cas9 er der i sorten Stairway induceret en deletion på 2 basepar (bp) i genet CI-1A. Genet bliver således ikke udtryk da deletionen indfører et frame-shift. Der er ikke transgene eller cisgene elementer i planten, idet CRISPR/Cas9 konstruktet er påvist ikke at være indsat i linjernes genom. CI-1A koder for en protease inhibitor. Mel/foder fra kerner fra den genom-redigerede linjer virker derved mindre inhiberende overfor proteaser, hvilket giver en bedre udnyttelse af protein i foderet.

i) Beskrivelse af de metoder der er anvendt

Genom-redigering af CI-1A i byg:

En beskrivelse af hele metoden er publiceret i:
Panting, M., Holme, I.B., Dionisio, G. and Brinch-Pedersen, H. (2025), Simplex and multiplex CRISPR/Cas9-mediated knockout of grain protease inhibitors in model and commercial barley improves hydrolysis of barley and soy storage proteins. Plant Biotechnol. J, 23: 2418-2428. https://doi.org/10.1111/pbi.70065

I korte træk er følgende metode brugt:

Byg af sorten Stairway er redigeret ved hjælp af vektoren pANIC6A der udtrykker et CRISPR/Cas9 kompleks. Som protospacer er brugt sekvensen (5'-3'): GAAGAACATGAGTTCCATGGAGG. Denne protospacer har genet CI-1A som mål. T-DNA er overført til byg ved hjælp af *Agrobacterium tumefaciens* AGL0. Denne udtrykker CRISPR/Cas9 komplekset som redigeret målgenet defineret af protospacer sekvensen. Som følge af redigeringen er identificeret en homozygot linje med en 2-bp deletion i målgenet. Deletionen på 2 bp er identificeret ved PCR hvor følgende primere er blevet anvendt til at amplificere genet CI-1A:

CI-1A fw	CTGTTTTCTTGCTTCCGT
CI-1A rv	CCATGGTATGCTGATGTT

Som giver et produkt på 891 bp. Dette er følgende blevet sekventeret ved sanger sequencing.

Ved udspaltning er der opnået en linje som ikke indeholder transgene elementer fra vektoren pANIC6A. Dette er muligt fordi T-DNA indsættes et vilkårligt sted i genomet som derfor i de fleste linjer ikke er samme sted som der hvor selve genomredigeringen sker. Tilstedeværelse/fravær af indsæt fra vektoren er detekteret ved PCR hvor følgende primere er anvendt:

Hyg fw	ACTCACCGCGACGTCTGTCG
Hyg rv	GCGCGTCTGCTGCTCCATA

Disse giver et forventet produkt på 727 bp.
Den i Panting et al. 2025 linje med navnet Stairway-ci-1a #24 er den som i dette forsøg ønskes udsat.
Kun denne linje ønskes udsat.

ii) Den anvendte vektors art og oprindelse

Vektoren anvendt til genomredigering (pANIC6A – appendix 1) indeholder elementer til selektion og komponenter til CRISPR/Cas9 komplekset.

Som beskrevet ovenfor indeholder den genomredigerede byg **ikke** elementer fra den anvendte vektor.

iii) Kilden til den/de til transformationen anvendte nukleinsyre(r) samt størrelse og tilsigtet funktion af hver bestanddel af den region, der skal indsættes

Det understreges igen at den genomredigerede byg ikke indeholder elementer fra den anvendte vektor.

b) Oplysning om GMHP'erne

j) Overordnet beskrivelse af de egenskaber og karakteristika, der er indført eller ændret

Linjen udtrykker ikke protease inhibitoren CI-1A. Foder fra planten giver derfor en bedre udnyttelse af protein og kvælstof ved proteolytisk nedbrydning af foderets proteiner.

ii) Oplysninger om faktisk indsatte/deleterede sekvenser

Det understreges igen at den genomredigerede byg ikke indeholder elementer fra den anvendte vektor. I målgenet CI-1A er følgende 2 bp deleteret (understreget):

GAAGAACATGAGTTCCATGGAGG

Linjen er homozygot for deletionen.

iii) Dele af Planten, hvori insertet udtrykkes

De 2 basepar i målgenet giver et frame-shift der fører til at målgenet CI-1A ikke udtrykkes noget sted i planten.

iv) Insertets genetiske stabilitet og GMHP'ernes fænotypiske stabilitet

Der er ikke indsat noget genetisk element i byg planterne. Da den genomredigerede ændring på 2-bp deletion i målgenet CI-1A nedarves som homozygoter ved selvbestøvning vil planterne have fuld fænotypisk stabilitet ved fremtidige generationer.

Der er desuden udført molekylære karakteriseringer for en detaljeret dokumentation af fravær af hygromycin resistens gen gennem flere generationer, samt vurdering af metodens detektionsgrænse med brug af kontroller. Dette forefindes i appendix 3.

c) Konklusioner af den molekylære karakterisering

En deletion på 2 basepar er induceret i målgenet. Der er ikke fundet tegn på at uønskede dele af den anvendte plasmid-vektor er indsat i den anvendte linje.

Vi vurderer ikke byg planterne under NGT-forordningen (EU) 2025/627 idet vi antager at planterne alligevel bliver vurderet og skal håndteres under den gældende GMO forordning.

Litteratur med direkte relevans for linjerne

Panting, M., Holme, I.B., Dionisio, G. and Brinch-Pedersen, H. (2025), Simplex and multiplex CRISPR/Cas9-mediated knockout of grain protease inhibitors in model and commercial barley improves hydrolysis of barley and soy storage proteins. Plant Biotechnol. J, 23: 2418-2428. https://doi.org/10.1111/pbi.70065

B.3 Oplysninger om specifikke risikoområder

(se desuden uddybende beskrivelse i medsendte bilag 1, Miljørisikovurdering M5-D2)

a) Eventuelle ændringer i GMHP'ernes persistens...

Der forventes ingen ændringer i hverken persistens eller invasionsevne, ej heller i evnen til at overføre genetisk materiale til beslægtede plantearter.
(se desuden afsnittet *'Generel betragtning vedr. risiko for spredning'*)

b) Eventuelle ændringer i GMHP'ernes evne…

Der forventes ingen ændringer i evnen til at overføre genetisk materiale til mikroorganismer.
(se desuden afsnittet *'Generel betragtning vedr. risiko for spredning'*)

c) Vekselvirkningsmekanisme mellem GMHP'erne og målorganismerne…

De genomredigerede bygplanter har i drivhusforsøg vist ikke at være mere modtagelige for plantesygdomme end tilsvarende ikke-genomredigerede bygplanter af sorten Stairway.

d) Potentielle ændringer i GMHP'ernes vekselvirkninger…

Der forventes ingen ændringer.

e) Potentielle ændringer i landbrugspraksis…

Der forventes korn med en forbedret kvalitet som foder som bedre udnytter proteinoptag og derved mindsker udledning af kvælstof fra landbrugsproduktionen.

f) Potentielle vekselvirkninger med det abiotiske miljø…

Der forventes ingen påvirkninger på de abiotiske miljøer.

g) Oplysninger om enhver toksisk, allergenisk…

Der forventes ingen toksisk, allergisk eller anden skadelig påvirkning på menneskers eller dyrs sundhed som følge af de genetiske ændringer.

h) Konklusioner vedrørende de specifikke risikoområder

Der forventes ingen øget risiko for miljøpåvirkning, hverken på menneskers, dyrs sundhed eller omkringliggende natur, idet det ikke planlægges at bruge plantemateriale til fodder eller føde.
Alle dele af alle bygplanter anvendt i forsøget transporteres til og fra GMO godkendte (klasse: planter) faciliteter som forefindes på matriklen Flakkebjerg. Transporten foregår ved GMO godkendte forskrifter. Transporten foretages af personer med GMO kørekort.

B.4 Oplysninger om kontrol, overvågning og efterbehandling- og affaldshåndteringsplaner

B.4.a. Trufne forholdsregler

i) Afstand fra krydsningskompatible plantearter, både beslægtede vilde plantearter og afgrøder.

Der vil være mindst 15 m til nærmeste anden mark.

ii) Forholdsregler for at mindske/undgå spredning af de modificerede planters reproduktionsorganer (F.eks. Pollen, frø).

Høstede cisgenetisk modificerede byg planter og bygfrø vil blive opsamlet i dobbelt lukkede stof- eller plastposer, mærket med GMO, og transporteres i kasser til GMO godkendt laboratorium, som forefindes på adressen (Se bilag).
Alt plantemateriale af bygplanter bliver destrueret.
Efter brug vil poser og kasser blive rengjort og desinficeret.

B.4.b. Metoder til efterbehandling af stedet efter udsætning

Året efter det samlede forsøgs afslutning, vil de i sædskiftet anvendte parceller ligge som sort jord med månedlige harvninger (april til september) og overvågning.
Arealet vil blive overvåget ved observation af personale med GMO kørekort. i min. 4 år eller til der ikke længere findes spildplanter eller spildfrø. Hvis der findes blot én plante eller ét spildfrø, anses området for ikke ryddet. Lederen af markpersonalet har ansvaret for at observationerne gennemføres, og at evt. uregelmæssigheder indberettes til miljøstyrelsen og landbrugsstyrelsen. Ligeledes vil miljøstyrelsen og landbrugsstyrelsen modtage notifikation om forsøgets afslutning, når observationsperioden på 4 år er gået.
Arealet forventes udlagt med slåningsbrak fra forsøgets afslutning, som kan slås og overvåges. Området anses for at være ryddet når der i mere end 1 år ikke har været fundet hverken byg frø eller spirede byg planter på forsøgsarealet.

I byg parcellerne vil der i perioden fra høst af byg og til såning af lucerne/vikke blive undersøgt for planter fra eventuelle byg spildfrø. Disse behandles med herbicid. Der vil før såning af lucerne/vikke altid blive behandlet med herbicid.

Nedenfor er vist en forventet placering af forsøgsarealet i Flakkebjerg i 2026-2028 i markblok 651133-27, hvor der ikke er overlap til tidligere GMO-forsøgsarealer.

4.C Behandlingsmetoder, efter udsætning, herunder affald

Under dyrkningen: normal plantebeskyttelse imod ukrudt, skadedyr og andre sygdomme. Der gødes desuden med fast eller flydende kemisk kunstgødning. Der vandes efter behov.

Korn opbevares og transporteres i stofsække i forseglede plastkasser med låg. Mærket med GMO.

De høstede materialer vil blive transporteret i dobbelt lukkede plastposer mærket med GMO, placeret i kasser.
Efter brug vil kasser blive rengjort og desinficeret, og sække vil blive kørt til forbrænding/deponeret.
Lucerne/vikke anvendes udelukkende til jordforbedring og vil ikke blive transporteret fra området.

GMO affald håndteres som klinisk risikoaffald og destrueres ved forbrænding hos godkendt operatør.
Se desuden bilag 2, vdr. godkendt plan for håndtering af GMO affald.

4.D Overvågningsplaner og teknikker

Udsætningsmarken vil blive observeret hver uge i vækstperioden, og væksten vil blive noteret og beskrevet. Det observeres om der er tegn på spredning af byg frø eller plantedele fra parceller. I tilfælde af utilsigtet spredning vil spredte frø og hvis, det vurderes nødvendigt, hele forsøget, blive termineret. Efter høst og i årene efter (jf. pkt.4b) vil udsætningsmarken blive nøje overvåget for eventuelle nyligt spirrede byg planter.
Eventuelle planterester vil blive destrueret.

4.E Beredskabsplaner

Der forventes ikke krisesituationer med mulig undtagelse af potentielle hærværksaktioner, hvilket der ikke er tradition for i Danmark.
Lokaliteten vil blive overvåget med jævne mellemrum. Der vil blive opsat skilte forskellige steder ved marken, der beskriver forsøget samt navne og telefonnumre på de ansvarlige for forsøget: Kim Hebelstrup og Anders Vestergaard. Ved uregelmæssigheder følges samme procedures om beskrevet i 4.D samt i B.4.b

4.F Metoder og procedurer til beskyttelse af stedet

i)

Alle maskiner bliver rengjorte på det godkendte område med trykluft og vand (8 bar). Rengøringen sker med mindst 15 meter til nærmeste areal med enten kartofler eller byg. Vand fra rengøring nedsiver på det anvendte område.
Al transport til og fra mark vil ske i lukkede enheder/kasser, hvorfor risiko for spredning under transport også betragtes som minimal.

ii)

Der forventes ikke at skulle gøres noget ekstra til beskyttelse af stedet mod uvedkommende personers indtrængen.

iii)

Der forventes ikke at skulle gøres noget ekstra til beskyttelse af stedet mod andre organismers indtrængen.

B.5 Beskrivelse af teknikker til påvisning og identifikation af GMHP´erne

Det redigerede gen indeholder en deletion på 2 basepar. Dette kan identificeres ved PCR og sanger sekventering som beskrevet i Panting et al. 2025. Alternativt kan sekventeringen erstattes af *i*ndel detection by amplicon analysis (idaa).

B.6 Oplysninger om tidligere udsætninger af GMPH´erne

Ikke relevant.

Underskrifter

Kim Hebelstrup, Aarhus Universitet

Anders Vestergaard, Aarhus Universitet

Bilag:

1. **Miljørisikovurdering**
2. **Aarhus Universitet, Flakkebjergs GMO-godkendelse af laboratorie.**
3. **Foreløbig skitse til forsøgsplaner i marken samt oversigtskort af ansøgte områder**
4. **Beskrivelse af maskiner**

Appendix:

1. Map af anvendt plasmid til CRISPR/Cas9 i byg
2. DNA sekvens af plasmid anvendt i byg
3. Dokumentation for fravær af hygromycin resistensgen gennem flere generationer

Bilag 1
Miljørisikovurdering

Ansøgning om brug af genomredigeret byg i sædskifteforsøg.

M5 –D2: I tilfælde af genetisk modificerede højerestående planter (GMHPer)

1. **Persistens og invasionsevne hos GMHPerne, herunder genoverførsel fra plante til plante.**
   A)
   Byg er selvbestøvende og der er således generelt ikke risiko for genoverførsel til andre bygplanter. Vi understreger, at der altid er en afstand på mindst 15 meter til nærmeste ikke-klassificerede område. Da byggen i nærværende ansøgning hverken indeholder transgener eller cisgener er der ikke mulighed for spredning af transgener eller cisgener til andre planter.

   B)
   Der er en begrænset risiko for spildfrø på de anvendte parceller. Efter høst ligger jorden sort og eventuelle byg planter vil blive nedsprøjtet eller fjernet manuelt.

   Året efter forsøgenes afslutning vil arealerne ligge som sort jord med månedlige harvninger (april til september) og overvågning. Arealet vil blive overvåget i min. 4 år eller til der ikke længere findes Spildplanter.
   Arealerne forventes udlagt med slåningsbrak fra 2029.

2. **Genoverførsel fra plante til mikroorganismer**
   Vurderes som værende uden betydning og er ikke kendt i byg.

3. **GMHPernes vekselvirkning med målorganismer**
   Der er ikke en målorganisme for forsøgene med byg.

4. **GMHPernes vekselvirkning med ikke-målorganismer**
   Det vurderes ikke at de udsatte planter vil have påvirkning på ikke-målorganismer

5. **Virkningerne af de specifikke dyrknings-, håndterings- og høstteknikker.**
   Det vurderes, at arbejdet i forbindelse med såning, høst og rengøring af maskiner sikrer en meget høj grad af sikkerhed for at der ikke efterlades spildfrø eller planter i jorden.

   Transport til og fra mark vil foregå i dobbelt lukkede enheder. Al transport og håndtering vil foregå med de relevante personer, altså ingen eksterne transportører.

   De personer, som skal foretage de kritiske arbejdsopgaver, transport, såning, høst og efterkontrol vil alle have taget GMO kørekort når forsøgene igangsættes, hvorfor alle er opdateret med nyeste viden om emnet.

6. **Virkninger på biogeokemiske processer**
   Det forventes ikke at byggen vil have indvirkning på biogeokemiske processer.

7. **Virkninger på menneskers og dyrs sundhed**
   Det vurderes, at byggen vil have end bedre udnyttelse af protein, når anvendt som dyrefoder. I tillæg skal det her nævnes, at der ikke er planer om sådanne forsøg med dyrefoder.

Arbejdstilsynet

Arbejdstilsynet
Tilsynscenter Øst
Landskronagade 33
2100 København Ø

T 70 12 12 88
at@at.dk
www.at.dk

CVR 21481815

11. december 2023

Sag
20230085859/2
Ansvarlig:
Rikke Kolding Hansen

P 1017874450

Aarhus Universitet
Nordre Ringgade 1
8000 Aarhus C

Afgørelse om afmelding af klassifikation til genteknologisk arbejde klasse planter

Arbejdstilsynet har den 3. december 2023 modtaget anmeldelse fra Forskningscenter Flakkebjerg, Aarhus Universitet ved Inger Holme (inger.holme@agro.au.dk) vedrørende afmelding af lokalerne P3A3, P4B1, P4P2 i klassifikation til genteknologisk arbejde klasse planter, LAB-id 214 175 beliggende Forsøgsvej 1, 4200 Slagelse. Ansvarlig laboratorieleder er angivet til Rikke Jakobsen.

Anmeldelsen er fremsendt i henhold til Arbejdstilsynets bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008.

Beskrivelse
Virksomheden har fremsendt ´Anmeldeskema til ændringer af klassifikation´. Virksomheden oplyser, at lokalerne er omhyggeligt rengjorte efter endt anvendelse til GM-planter samt at følgende lokaler vil indgå i lab id'et fremadrettet: P4A1, P4A2, P4SA.

Vurdering
Arbejdstilsynet finder på det foreliggende grundlag, at de omhandlede lokaler kan afmeldes genteknologi klassifikationen.

Afgørelse
Arbejdstilsynet meddeler hermed forsat klassifikation til genteknologisk arbejde klasse planter i lokale P4A1, P4A2, P4SA (fællesgang) samt 20 plastkasser i rum E 128 samt V02 beliggende Forskningscenter Flakkebjerg, Aarhus Universitet, Forsøgsvej 1, 4200 Slagelse, jf. § 7, stk. 1, i Arbejdstilsynets bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008.

Lokalerne har forsat LAB-id nr. 214 175

Vejledning
Opmærksomheden henledes på, at det af hensyn til klassifikationen er vigtigt at sikre, at forskrifter, procedurer m.v. fortsat afspejler de faktiske sikkerhedsmæssige forhold for arbejdet med GMO, herunder arbejdsmetoder og arbejdsgange. Ved at gennemgå dem med jævne mellemrum, fx i forbindelse med revideringen af virksomhedens APV kan dette sikres.

Opmærksomheden henledes endvidere på § 30 jfr. § 11 i bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008, hvorefter enhver væsentlig ændring af de oplysninger, der ligger til grund for denne klassifikation, skal anmeldes til Arbejdstilsynet.

Ligeledes henledes opmærksomheden på § 12 i samme bekendtgørelse, hvorefter det forinden skal anmeldes til Arbejdstilsynet, hvis klassifikationen ikke længere ønskes opretholdt.
Med henblik på en senere evt. afmelding af klassifikationen kan Arbejdstilsynet anbefale, at virksomheden allerede nu, udarbejder en skriftlig nedklassificeringsprocedure.

Klage
Klage over afgørelsen skal indsendes til Arbejdstilsynet inden 4 uger fra afgørelsens dato.

Kopi af dette brev er sendt til Miljøstyrelsen (J.nr. MST-686-00044), Tolderlundsvej 5, 5000 Odense C.

Venlig hilsen

Rikke Kolding Hansen

Arbejdstilsynet

Arbejdstilsynet
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CVR 21481815

16. oktober 2019

Sag
20180028531/14
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Afgørelse om ændringer af klassifikation til genteknologisk arbejde klasse 1 og planter

Inger Holme, Dept. of Molecular Biology and Genetics, Aarhus University, Forsøgsvej, 4200 Slagelse, e-mail; inger.holme@mbg.au.dk har med henvendelse af 2. september 2019 søgt om ændring af klassifikation til genteknologisk arbejde klasse 1 og planter – lab id 214 167.

Der anmodes om afklassificering af lokalerne B242 og autoklave i B228, begge blevet grundigt rengjort, således at der ikke findes rester af GMO samt anmodes om udvidelse med lokalerne B128, B130, A010, A030 og E224.

Ansøgningen er fremsendt i henhold til Arbejdstilsynets bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008.

Beskrivelse
Virksomheden har fremsendt udfyldt skema ”Anmeldelse til klassifikation af genteknologiske laboratorier og laboratorieområder samt anlæg til genteknologiske stor-skalaforsøg eller produktion”.

Ansøgningen har været forelagt Miljøstyrelsen(MST-686-00054), som den 15. oktober har sendt følgende bemærkninger:

”Miljøstyrelsen foretog besigtigelse af de lokaler der ønskes udvidet med den 28. august 2019 i forbindelse med et tilsyn. Lokalerne er indrettet ligeledes beskrevet i tidligere ansøgninger og som allerede godkendte lokaler.

Miljøstyrelsen vurderer, at de oplyste sikkerhedsrutiner og etablerede indeslutningsforanstaltninger yder en effektiv sikring imod at genetisk modificerede planter og mikroorganismer spredes til det ydre miljø.

Styrelsen har på det foreliggende grundlag ikke indvendinger mod den søgte klassifikation om ændringer af lab id 214 167.”

Vurdering
Arbejdstilsynet finder på det foreliggende grundlag, at de omhandlede lokaler, sikkerhedsforskrifter m.m. lever op til de krav, der er gældende for genteknologisk arbejde klasse 1 og planter.

Afgørelse
På baggrund af ovenstående meddeler Arbejdstilsynet hermed klassifikation til genteknologisk arbejde klasse 1 og planter i lokale A010, A030, B108, B110, B128, B130, B132, B138, B139, B140, B153, B155, B161, B244, B246, autoklave i lokale D108, E224 samt tæskemaskine i laden i lokale E101 beliggende Forskningscenter Flakkebjerg, 4200 Slagelse jf. § 7, stk. 1, til Arbejdstilsynets bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008.

Lokalerne har fået tildelt lab. id. nr.: 214 167

Denne afgørelse erstatter afgørelse af den 8. november 2018.

Vejledning
Opmærksomheden henledes på, at det af hensyn til klassifikationen er vigtigt at sikre, at forskrifter, procedurer m.v. fortsat afspejler de faktiske sikkerhedsmæssige forhold for arbejdet med GMO, herunder arbejdsmetoder og arbejdsgange. Ved at gennemgå dem med jævne mellemrum, fx i forbindelse med revideringen af virksomhedens APV kan dette sikres.

Opmærksomheden henledes endvidere på § 30 jfr. § 11 i bekendtgørelse om genteknologi og arbejdsmiljø nr. 910 af 11. september 2008, hvorefter enhver væsentlig ændring af de oplysninger, der ligger til grund for denne klassifikation, skal anmeldes til Arbejdstilsynet.

Ligeledes henledes opmærksomheden på § 12 i samme bekendtgørelse, hvorefter det forinden skal anmeldes til Arbejdstilsynet, hvis klassifikationen ikke længere ønskes opretholdt.
Med henblik på en senere evt. afmelding af klassifikationen kan Arbejdstilsynet anbefale, at virksomheden allerede nu, udarbejder en skriftlig nedklassificeringsprocedure.

Klage
Klage over afgørelsen skal indsendes til Arbejdstilsynet inden 4 uger fra afgørelsens dato.

Kopi af dette brev er sendt til MILJØSTYRELSEN, Tolderlundsvej 5, 5000 Odense C.

Venlig hilsen

Rikke Kolding Hansen

Udgave af 1. marts, 2007

SIKKERHEDSFORSKRIFTER FOR GENTEKNOLOGISK ARBEJDE I KLASSIFICEREDE LABORATORIER VED FORSKNINGSCENTER FLAKKEBJERG

1. Uvedkommende personers adgang til laboratoriet skal begrænses.

2. Der skal bæres kittel eller andet egnet særligt arbejdstøj i laboratoriet.

3. Tobak, føde- og drikkevarer må ikke nydes i laboratoriet.

4. Det særlige arbejdstøj aftages inden spisning samt ved arbejdstids ophør.

5. Der skal udarbejdes procedure for transport af biologisk aktivt materiale ud af laboratoriet, og om hvorledes mærkning af transportbeholdere skal foretages.

   Proceduren er følgende: Transport indenfor bygningen fra et klassificeret laboratorium til et andet skal ske i lukkede beholdere, der er mærket med gul tape med påskriften "Biologisk aktivt materiale". I det efterfølgende vil betegnelsen "mærket med gul tape" referere til tape med denne påskrift.

6. Alt affald med indhold af biologisk aktivt materiale skal opsamles i egnede beholdere og autoklaveres.

   Egnede beholdere er for flydende affald autoklaverbare glasflasker med skruelåg. For fast affald anvendes rustfri, runde stålspande eller plastspande med et tætsluttende låg, herefter benævnt autoklavespande. Begge typer beholdere mærkes med gul tape, jvf. punkt 5. Dersom ingen autoklave er ledig ved arbejdets ophør, anbringes affaldsbeholderne i et stinkskab.

7. Når affaldet med indhold af biologisk aktivt materiale skal transporteres udenfor det klassificerede område for at blive autoklaveret, skal det transporteres i autoklavespande.

8. Kanyler, engangssprøjter, skarpe genstande og lignende anbringes i autoklaverbare glasflasker med skruelåg, mærket med gul tape.

9. Utensilier, glasvarer og lignende, forurenet med biologisk aktivt materiale, skal autoklaveres før rengøring.

   Se forklaringen til punkt 6. ovenfor.

10. Særligt arbejdstøj, der er forurenet med biologisk aktivt materiale, desinficeres med 70% ethanol, hvorefter det sendes til normal vask.

11. Hvis der foregår forsøg eller lignende uden tilstedeværelse af sagkyndige, skal det være muligt at tilkalde en fagligt kompetent person.

    De private tlf. numre på Preben Bach Holm og Annemarie Fejer Justesen er angivet sidst i sikkerhedsforskriften

12. Der skal føres logbog over alle uheldssituationer, der har givet anledning til risiko for personers sikkerhed eller sundhed eller for det ydre miljø. Disse logbøger opbevares hos Preben Bach Holm (rum E214) og Annemarie Fejer Justesen (rum B241)

13. Arbejdet skal udføres, så dannelse af aerosoler begrænses mest muligt.

    Særligt aerosoldannende operationer er f.eks. bobling af bakteriekulturer, brug af blender, sonikering, forcering af væske gennem en kanyle.

14. Mundpipettering er ikke tilladt.

15. Arbejdet skal udføres i kabinet udstyret med eget ventilationssystem, når der er fare for sundhedsskadelig luftforurening.

    Bakterie- og gærkulturer håndteres i en vertikal-flow sikkerhedsbænk, hvorimod transgene plantecellekulturer kan håndteres i en horisontal-flow bænk

16. Kanyler, sprøjter, skarpe genstande og lignende skal anvendes mindst muligt.

17. Arbejdspladser i laboratoriet skal rengøres og desinficeres dagligt.

    Laboratorieborde skal desinficeres og rengøres dagligt af de personer, der udfører arbejdet. Rengøringspersonalet må ikke rengøre apparatur, kemikalieflasker m.m. samt bordflader og hylder, der er blokeret heraf. Derfor påhviler det den enkelte, der har en arbejdsplads i laboratoriet, eller som har ansvar for et fællesrum, at udføre eller foranledige sådan rengøring. For at lette dette arbejde, anbefales det jævntligt at frigøre hele hylder, således at rengøringspersonalet kan komme til. Hvert år i januar måned foretages en hovedrengøring, idet hylder m.v. ryddes, og rengøringen aftales med rengøringspersonalet. Supplerende rengøring foretages af de personer, der arbejder i rummet.

18. Arbejdspladsen skal holdes ryddelig.

19. Der skal findes egnede desinfektionsmidler i laboratoriet.

20. Ved spild af biologisk aktivt materiale skal der straks desinficeres med 70% ethanol.

21. Egnede handsker skal anvendes ved kontakt med biologisk aktivt materiale.

22. Biologisk aktivt materiale må ikke hældes i afløb/kloak.

    Materialet skal inaktiveres ved autoklavering eller ved spild og uheld med 70% ethanol. Husk ved store volumener og viskøse væsker, etc. at forøge tiden for autoklaveringen.

Annemarie Fejer Justesen: **Tlf. 58 14 30 27**
Preben Bach Holm: **Tlf. 38 71 37 62**

1 marts, 2007

Preben Bach Holm

Udgave af 1. marts, 2007

SIKKERHEDSFORSKRIFTER FOR DYRKNING AF TRANSGENE PLANTER I KLASSIFICEREDE DRIVHUSE VED FORSKNINGSCENTER FLAKKEBJERG

1) Generelt

Alle dyrkninger af transgene planter i de klassificerede drivhuse meddeles til seniorforsker Preben Bach Holm (D106, tlf. 3649) eller seniorforsker Henrik Brinch-Pedersen (B239, tlf. 3651), der også er med til at planlægge det praktiske drivhusarbejde. Eventuelle problemer med spild og andre uheld meddeles Preben Bach Holm, der registrerer disse i en logbog.

2) Identifikation og mærkning af transgene planter

Alle transgene planter skal kunne identificeres entydigt fra ikke transgene planter. Udover betegnelser og numre refererende til plantens oprindelse og karakteristika skal alt transgent materiale plantet i potter mærkes med gul tape med teksten "Biologisk aktivt materiale". Ved plantning direkte i jord skal området med de transgene planter afgrænses og afmærkes på en passende måde.

3) Klassificerede drivhuskabiner

Følgende drivhuskabiner er klassificerede til dyrkning af transgene planter

P4A1, P4A2
P4B1, P4B2

4) Adgang til drivhuskabiner

Adgangen til drivhuskabinerne skal begrænses mest muligt. Dørene til kabinerne forsynes med et gult skilt med teksten "Genteknologisk laboratorieområde, Klasse 1".

5) Plantering

Al plantering af transgene frø eller planter regenereret fra vævskultur foregår på et bord i kabine P4B. Alle planteringer aftales med gartner Ole Braad Hansen.

6) Transport af planter

Under transport af planterne skal blomster- og frøbærende dele af disse dækkes af pollentætte poser for at hindre spredning af pollen og frø.

7) Håndtering af planterne under blomstring og frøsætning

Planter som hvede og byg blomstrer under drivhusbetingelser mens akset stadig er omgivet af blade. I tilfælde af åben blomstring efter aksskridning dækkes enten de enkelte aks eller hele planten med pollentætte poser. Alle åbentblomstrende arter, og i særdeleshed vindbestøvende arter som rajgræs, **skal** dækkes af pollentætte poser under blomstring.

8) Høstning af frø

Aks og blomsterstande kan afskæres på stedet men den videre behandling (udpilning af frø) skal foregå på planteringsbordet i kabine P4B2 eller i et af de klassificerede laboratorier. Når tærskning af større partier kommer på tale skal der udarbejdes en særskilt sikkerhedsinstruks for denne proces.

9) Håndtering af affald

Efter høstning af frøene anbringes det vegetative affald i en særlig aflåst kontainer, mærket med teksten "Biologisk aktivt materiale". Planteaffaldet sendes derefter til en forbrændingsanstalt.

10) Biologisk indeslutning

Det tilstræbes, i det omfang det overhovedet er muligt, at der i hver enkelt område kun dyrkes enten transgene eller ikke transgene planter. Efter endt dyrkning rengøres dyrkningsbordene omhyggeligt før en ny dyrkningscyklus påbegyndes. I tilfælde af, at der dyrkes transgene og ikke transgene planter af samme art i det samme område skal frø og affald behandles, som om de var transgene.

Private tlf. numre

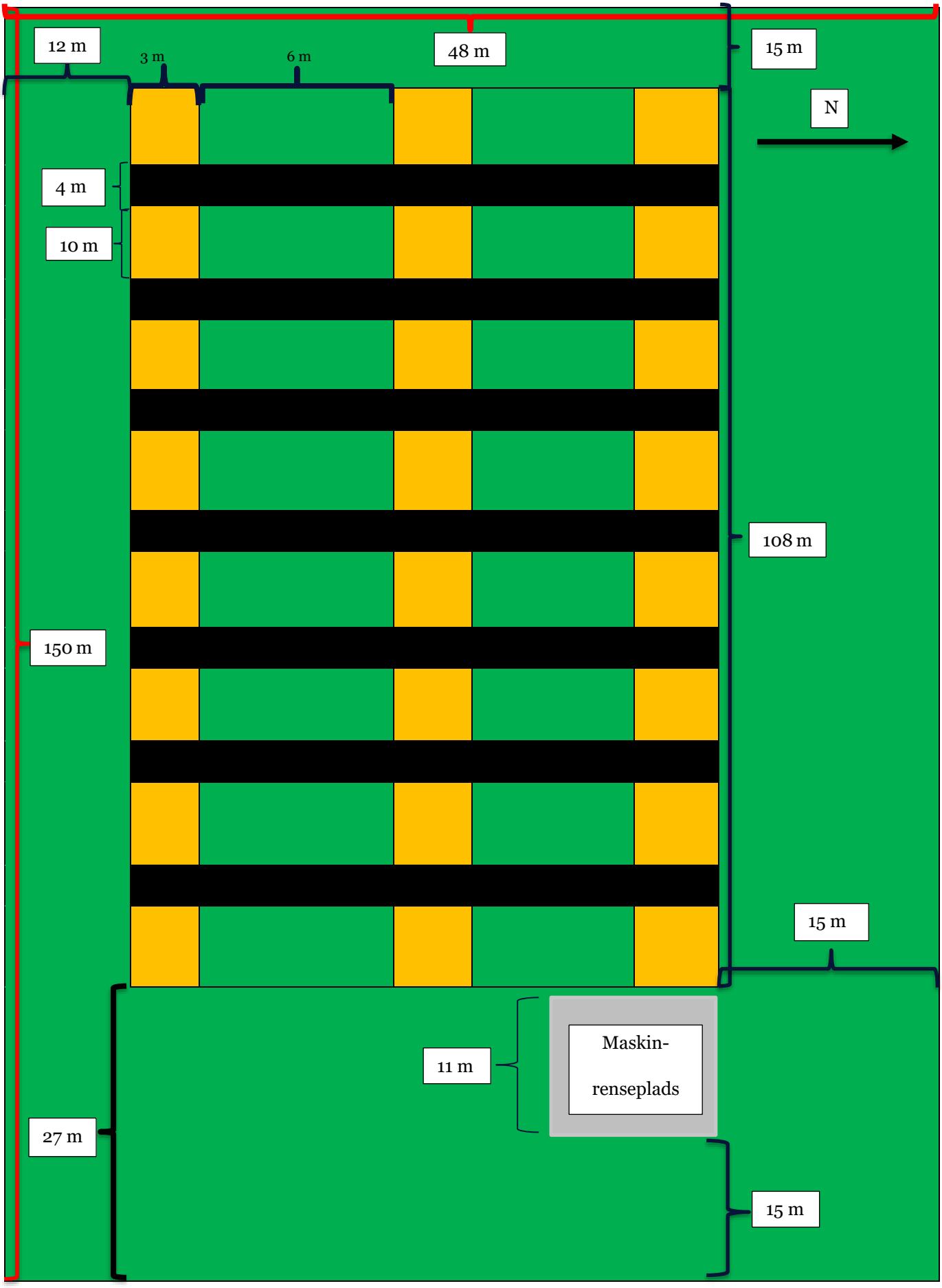
Henrik Brinch-Pedersen: **5819 1079**
Preben Bach Holm: **3871 3762**

1. marts 2007

Preben Bach Holm

Bilag 3 - Foreløbig skitse til forsøgsplaner i marken samt oversigtskort af ansøgte område (Flakkebjerg)

Skitse over forsøgsareal. De gule felter markerer parceller hvor afgrøderne vil blive dyrket. Grøn er græsarealer.



Arealets placering i forhold til nabo (35 meter) og §3 (mose) område (200 meter):

Oversigt af markblokke:

Bilag 4 – Beskrivelse af maskiner

Der vil blive anvendt en del forskellige traktorer til at udføre de maskinelle arbejdsopgaver.
(billeder ikke vedlagt)
Ford 7740, Case 130 CVX, Ford 3600, Ford 3140, Ford 6080

For tilberedning af jorden vil der blive anvendt to fræsere: en fræser med en arbejdsbredde a 1,5 meter (ovenstående billede).

Der vil til tilberedning af jorden også blive en anvendt en fræser af mærket Kuhn EL 92.

Haldrup C-85

Forsøgsmejetærsker til høst af byg.

Nedvandingssprøjte til behandling med herbicider, fungicider og øvrige plantebeskyttelsesmidler, vækstregulatorer samt flydende kemisk gødning.

Gødningsspreder til granuleret kemisk gødning

Kverkeland Accord

Kombineret såmaskine og såbedsharve

Scan agro – brakklipper til afpudsning af værnearealer og køregange

Horsch Terrano 3FX

Dybdeharve

Kongskilde Germinator

Forårsharve til forberedelse af såbed

Kaeser M26 Mobil kompressor

Bruges til at rense maskiner på det klassificerede område med trykluft

Tromle

Bruges kun ved behov

Hege 90 såmaskine

1,5 m bred såmaskine

JCB 525.60 teleskoplæsser til transport af materiel. Herunder til transport af NGT plantemateriale i dobbeltposer i aflukkede containere (som beskrevet i ansøgningen) mellem det anvendte markareal I Flakkebjerg og GM klassificerede laboratorier og faciliteter i Flakkebjerg.

Appendix 1 - **Map af anvendt plasmid til CRISPR/Cas9 i byg**

Appendix 2 - **DNA sekvens af plasmid anvendt i byg**

pANIC_6A-CR-Expression CI-1A vector sequence

CTCGAGGTCATTCATATGCTTGAGAAGAGAGTCGGGATAGTCCAAAATAAAACAAAGGTAA-
GATTACCTGGTCAAAAGTGAAAACATCAGTTAAAAGGTGGTATAAAGTAAAATATCGGTAATAAAAGGTGGC
CCAAAGTGAAATTTACTCTTTTCTACTATTATAAAAATTGAGGATGTTTTTGTCGGTACTTT-
GATACGTCATTTTTGTATGAATTGGTTTTTAAGTTTATTCGCTTTTGGAAATGCATATCTGTATTTGAGTCGG
GTTTTAAGTTCGTTTGCTTTTGTAAATACAGAGGGATTTGTATAAGAAA-
TATCTTTAAAAAAACCCATATGCTAATTTGACATAATTTTTGAGAAAAATATATATTCAGGCGAATTCTCACA
ATGAACAATAATAAGATTAAAATAGCTTTCCCCCGTTGCAGCGCATGGGTATTTTTTCTAG-
TAAAAATAAAAGATAAACTTAGACTCAAAACATTTACAAAAACAACCCCTAAAGTTCCTAAAGCCCAAAGTGC
TATCCACGATCCATAGCAAGCCCAGCCCAACCCAACCCAACCCAACCCACCCCAG-
TCCAGCCAACTGGACAATAGTCTCCACACCCCCCCACTATCACCGTGAGTTGTCCGCACGCACCGCACGTCTC
GCAGCCAAAAAAAAAAAAAGAAAGAAAAAAAAGAAAAAGAAAAAACAG-
CAGGTGGGTCCGGGTCGTGGGGGCCGGAAACGCGAGGAGGATCGCGAGCCAGCGACGAGGCCGGCCCTCC
CTCCGCTTCCAAAGAAACGCCCCCCATCGCCACTATATACAT-
ACCCCCCCCTCTCCTCCCATCCCCCCAACCCTACCACCACCACCACCACCACCTCCACCTCCTCCCCCCTCGCT
GCCGGACGACGAGCTCCTCCCCCCTCCCCCTCCGCCGCCGCCGCGCCGGTAAC-
CACCCCGCCCCTCTCCTCTTTCTTTCTCCGTTTTTTTTTTCCGTCTCGGTCTCGATCTTTGGCCTTGGTAGTTT
GGGTGGGCGAGAGGCGGCTTCGTGCGCGCCCAGATCGGTGCGCGGGAGGGGCGG-
GATCTCGCGGCTGGGGCTCTCGCCGGCGTGGATCCGGCCCGGATCTCGCGGGGAATGGGGCTCTCGGATGT
AGATCTGCGATCCGCCGTTGTTGGGGGA-
GATGATGGGGGGTTTAAAATTTCCGCCATGCTAAACAAGATCAGGAAGAGGGGAAAAGGGCACTATGGTTT
ATATTTTTATATATTTCTGCTGCTTCGTCAGGCTTAGATGTGCTA-
GATCTTTCTTTCTTCTTTTTGTGGGTAGAATTTGAATCCCTCAGCATTGTTCATCGGTAGTTTTTCTTTTCATG
ATTTGTGACAAATGCAGCCTCGTGCGGAGCTTTTTTGTAGGTAGACGATAA-
GCTTATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTC
CGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAG-
GAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGC
ACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTTAGCGA-
GAGCCTGACCTATTGCATCTCCCGCCGTTCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCC
CGCTGTTCTACAACCGGTCGCGGAGGC-
TATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCG
GTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGG-
CAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGA
GGACTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGAC-
GGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCG

CCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGC-
TACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCACGACTCCGGGCGTATATGCTCCGCATTGGTCTT
GACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCG-
CAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAG
CGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGAC-
GCCCCAGCACTCGTCCGAGGGCAAAGAAATAGAGTAGATGCCGACCGGGATCTGTCGATCGACAAGCTCGA
GTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCC-
TATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTAT
CAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTACTA-
AAATCCAGATCCCCCGAATTAATTCGGCGTTAATTCAGCTGCAGGTTTGGCCACTGGAGAGGGGCACACACG
TCAGTGTTTGGTTTCCACTAGCACGAGTAGCG-
CAATCAGAAAATTTTCAATGCATGAAGTACTAAACGAAGTTTATTTAGAAATTTTTTTAAGAAATGAGTGTAA
TTTTTTGCGACGAATTTAATGACAATAATTAATCGATGATTGCCTACAGTAATGCTACAG-
TAACCAACCTCTAATCATGCGTCGAATGCGTCATTAGATTCGTCTCGCAAAATAGCACAAGAATTATGAAATT
AATTTTACAAACTATTTTTATTTAATACTAATAATTAACTGTCAAAGTTTGTGCTACTCG-
CAAGAGTAGCGCGAACCAAACACGGCCTGGAGGAGCACGGTAACGGCGTCGACAAACTAACGGCCACCACC
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TTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCG
CATCGGGCTTCCCATACAATCGATAGATTGTCGCAC-
CTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCGG
CCTAGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTT-
GTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATCTGGATCACAGGCAGCAACGCTCTGTCATCG
TTACAATCAACATGCTACCCTCCGCGAGATCATCCGTGTTTCAAACCCGGCAGCTTAGTT-
GCCGTTCTTCCGAATAGCATCGGTAACATGAGCAAAGTCTGCCGCCTTACAACGGCTCTCCCGCTGACGCCG
TCCCGGACTGATGGGCTGCCTGTATCGAGTGGTGATTTTGTGCCGAGCTGCCGGTCGGG-
GAGCTGTTGGCTGGCTGGTGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACAC
ATTGCGGACGTTTTTAATGTACTGAATTAACGCCGAATTGCTCTAGCCAATACGCAAAC-
CGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCA
GTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACAC-
TTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGAC
ATGATTACGAATTCTTAATTAAGATATCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGAC-
CTGCAGGTTTGTACCGGGCCCCCC

Stairway ci-1a mutant for field trial

T_0 generation and analysis

Stairway mutant derived from ovule transformation without selection.

Total ovules transf.: 125; Total embryos: 98; Emb per 100 ovules: 78.4%. Emb germination: Plants per emb: 70/98=71.4%. Plant with roots per embryo:65/98=66.3%. Plants with roots per ovule (65/125): 52.0%. **Mutated: 1/65 embryos: 1.5%; 1/125 ovules=0.8%**

gDNA extraction from the single regenerated mutant line "29/3 no. 9". Sequences of the target mutation region showed a bi-allelic mutation in TOPO clones. One allele has a "CA" deletion in the 4th and 5th position upstream of the PAM site, while the other allele show a "CC" deletion in the 5th and 6th position upstream of the PAM site (Figure 1).

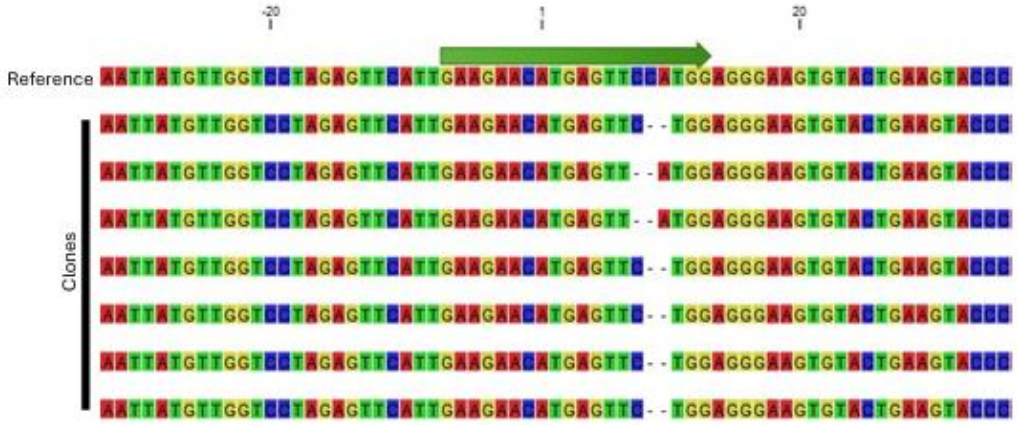

Figure 1. TOPO clone sequences from the "29/3 no. 9" T_0 mutant Stairway plant. A bi-allelic mutation of either CA or CC deletion is detected, when comparing to a wild type reference sequence. The green arrow indicates the 20 bp guide sequence.

In the following generation, it did however become clear that the T_0 plant regenerated was in fact a chimeric mutant, while there was T_1 plants having wild type alleles.

The following generations and analysis

From the "29/3 no. 9" T_0 plant, 20 T_1 plants were propagated. Genomic DNA was extracted and tested with two PCR reactions. PCR reaction one was an amplification of the target region, following a restriction enzyme digestion using NcoI. The restriction enzyme recognition site has been disrupted in the mutant alleles, meaning that wild type DNA is digested, leaving two fragments. Mutant alleles are not digested, leaving a single, intact band. Heterozygous plants have one allele digested and one intact,

resulting in all three bands (Figure 2). The second PCR amplifies a part of the hygromycin resistance gene. This gene is present in the integrated T-DNA which also includes the Cas9 gene and the guide RNA sequence. The hygromycin primers are common and used routinely in screening for the presence of T-DNA. The assay is a presence/non-presence of the PCR product and thus the T-DNA in the plant (Figure 2).

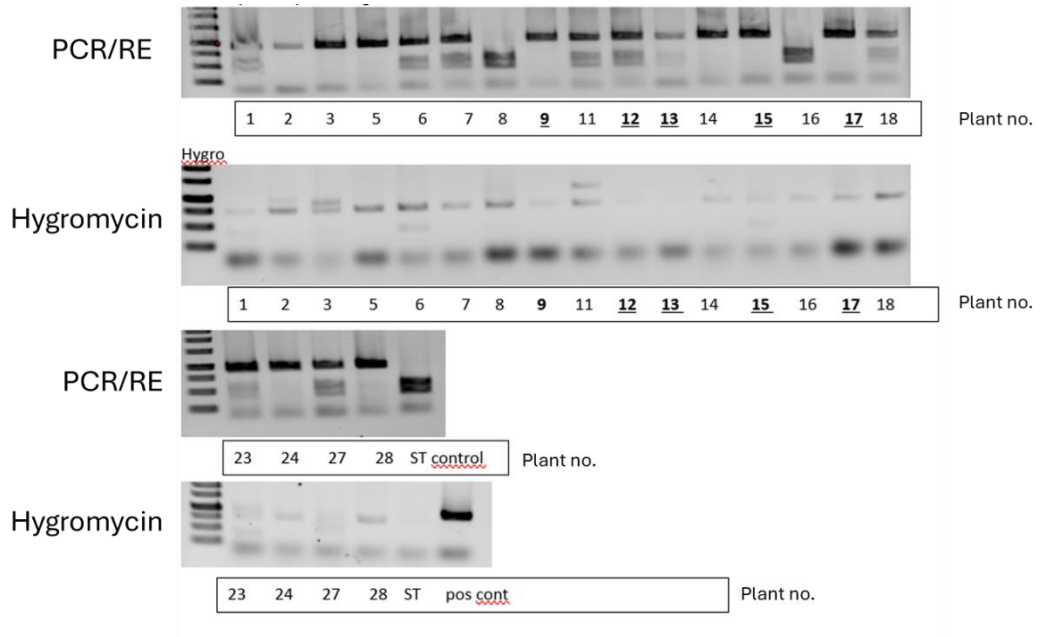

Figure 2. PCR/RE and PCR analysis of the mutation and T-DNA presence in T_1 plants. For the PCR/RE analysis, a stairway wild type control (ST control) has all of the PCR product digested with NcoI. For the hygromycin PCR screening of T-DNA presence, a Stairway wild type (ST) has no band/T-DNA, while the positive control (plasmid DNA) has a band.

Selected lines 2, 8, 12, 24 and 28 were TOPO cloned and sequenced (Figure 3). Included were homozygous (2, 24 and 28), heterozygous (12) and wild type (8) lines based on the PCR/RE analysis. The sequences confirm the PCR/RE results and also show that the "CC" mutation from the T_0 is the only mutation inherited in these lines.

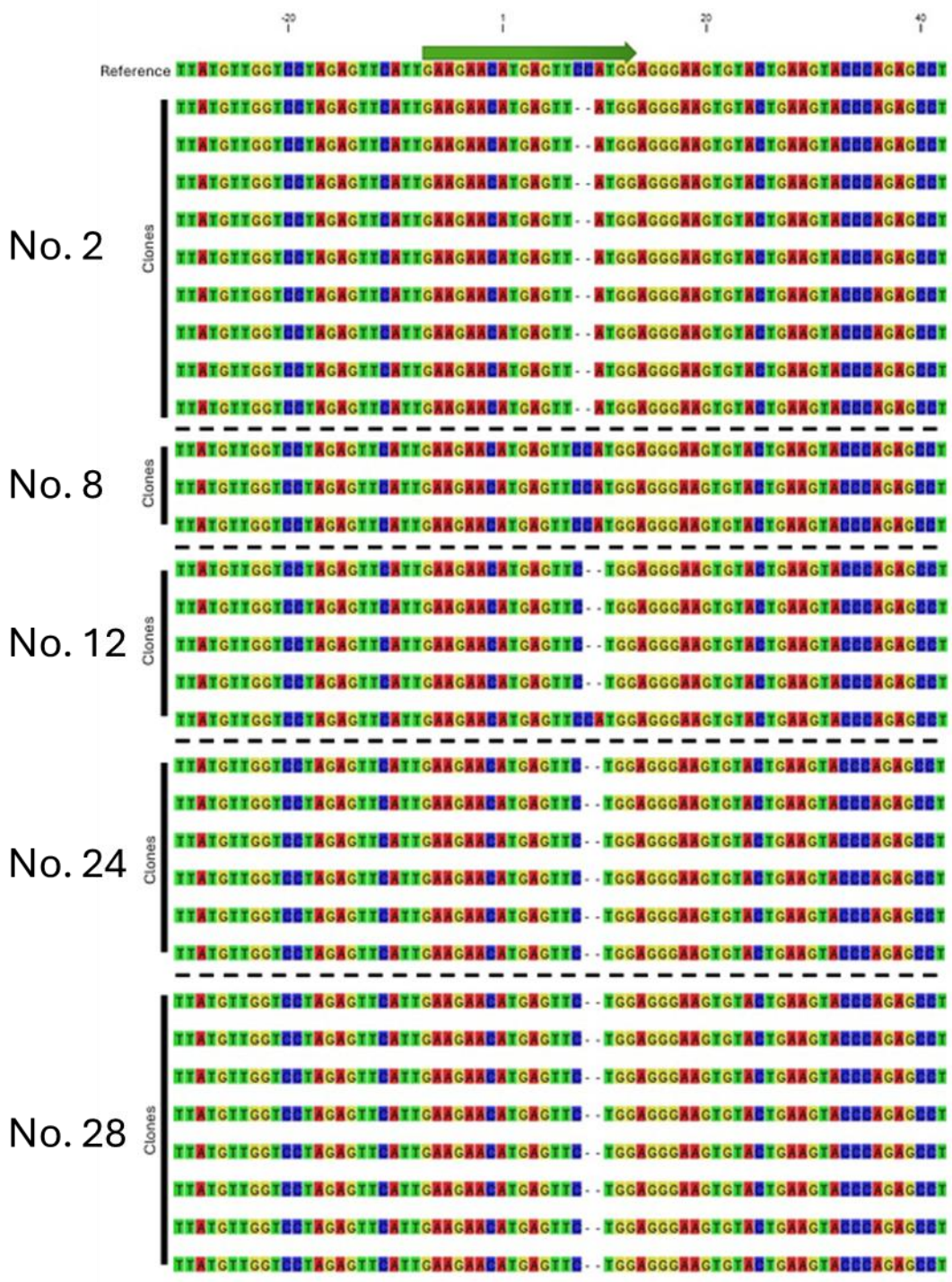

Figure 3. TOPO clone sequences of selected T_1 lines.

In the next generation (T_2), only plants from no. 24 was found without the T-DNA. Four lines (24-4, 24-6, 24-12 and 24-18) had no T-DNA and the T_3 progeny was also tested for the hygromycin gene and T-DNA insert to verify the absence. Ten seeds were sown from each line and DNA harvested and divided into two pools (A and B) for each line.

Germinations rates:		
Lines	**Germination/seeds**	**Germ. %**
24-4	10/10	100
24-6	8/10	80
24-12	9/10	90
24-18	8/10	80
wt	10/10	100

The DNA integrity was tested by amplifying the CI-1A gene. Secondly, the absence of the T-DNA was tested by the hygromycin PCR (Figure 4). The results show that the DNA integrity is OK for all of the plant samples with CI-1A band. As expected, the pANIC6A vector (were the T-DNA originates from) and the R40 (DNA buffer) show no bands with the CI-1A primer pair. All plants show no PCR band using the hygromycin primer pair, showing that the T-DNA is not present in any of the DNA pools (which includes DNA from 45 plants). There is a hygromycin band in the pANIC6A sample, while the R40 has no band, as expected.

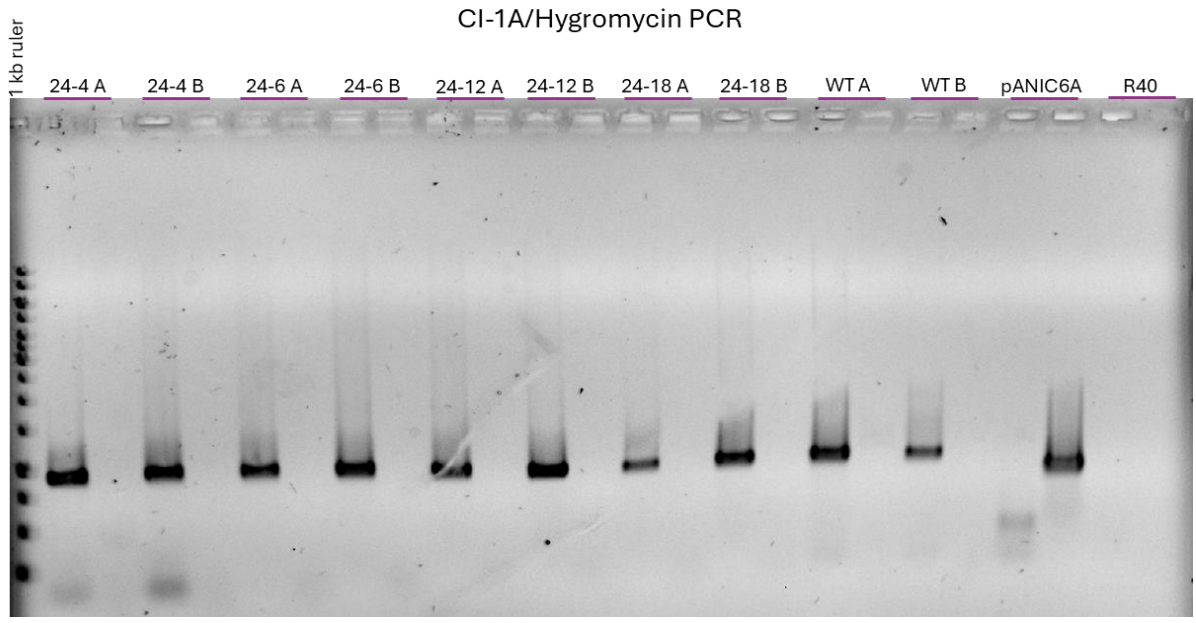

Figure 4. CI-1A and hygromycin PCR was run on pools of T_3 plants to verify the integrity of the DNA with a CI-1A gene band and for the abscense of the T-DNA in the plants by hygromycin PCR. As controls, two pools of Stairway wild type, the pANIC6A vector and R40 were included.

Agronomic traits were also evaluated on some of the plant at maturity. These results are also included in the published papers additional information (see table below and

Figure 5). There is no difference in the agronomical traits, except for the thousand kernel weight (TKW) being higher in the Stairway mutant.

Plant ID	Spikes per plant	Spike length (cm)	TKW (gram)
Stairway wt (n = 3)	42 ± 1	9.53 ± 1.58 (n = 111)	39.72 ± 4.15
Stairway ci-1a mut (n = 4)	42.75 ± 10.5	9.83 ± 1.57 (n = 157)	43.61 ± 6.08

Figure 5. Mature spikes of the Stairway mutant and the Stairway wild type.

T_4 generation

Seed propagation was done with the T_3 line determined 24-18-2, which is part of the screening above. These seeds are planned to be sown in field. The hygromycin gene and thus the T-DNA cassette has not been observed in any of the progeny since the T_2 generation.

Summary

Der er ikke fundet transgene elementer siden T_2 generationen, hvor der var fire linjer uden transgent insert. I T_3 generationen fandt vi ingen transgen i 45 testede planter.

Detektionsgrænsen er en til stede/ikke til stede af et PCR bånd med Hyg-primerne. Hvis hygromycingenet ikke er til stede længere, anses det som at hele T-DNA kassetten er

udpsaltet. De hygromycin primere der bruges til at screene for tilstedeværelsen af T-DNA er den metode som bliver brugt i laboratoriet.

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Simplex and multiplex CRISPR/Cas9-mediated knockout of grain protease inhibitors in model and commercial barley improves hydrolysis of barley and soy storage proteins

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Summary

Anti-nutritional factors in plant seeds diminish the utilization of nutrients in feed and food. Among these, protease inhibitors inhibit protein degradation by exogenous proteases during digestion. Through conventional and selection-gene-free genome editing using ovules as explants, we used simplex and multiplex CRISPR/Cas9 for studying the impact of chymotrypsin inhibitor CI-1A, CI-1B and CI-2, Bowman-Birk trypsin inhibitor, Serpin-Z4, and barley α-amylase/subtilisin inhibitor on barley and soybean storage protein degradation. Mutants were generated in the commercial cultivar Stairway, having a high level of protease inhibition, and the barley model cultivar Golden Promise, having a lower inhibition level. In Golden Promise, all individual knockouts decreased the inhibition of the three proteases α-chymotrypsin, trypsin and the commercial feed protease Ronozyme ProAct significantly. The triple knockout of all chymotrypsin inhibitors further decreased the inhibition of α-chymotrypsin and Ronozyme ProAct proteases. Degradations of recombinant barley storage proteins B- and C-hordeins were significantly improved following mutagenesis. In Stairway, a single knockout of CI-1A almost compares to the effect on the proteases achieved for the triple knockout in Golden promise, uncovering CI-1A as the major protease inhibitor in that cultivar. The Stairway mutant demonstrated significantly improved degradation of recombinant barley hordeins and in the soybean storage proteins glycinin and β-conglycinin. The results of this study provide insights into cereal protease inhibitor genes and their negative effects on the degradation of barley storage protein and the most important plant protein from soybeans. The study suggests a future focus on plant protease inhibitors as a major target for improving feed and food protein digestibility.

Introduction

Efficient protein digestibility is essential for a healthy diet and for reducing agriculture's impact on climate and the environment. In human diets, inefficient hydrolysis of certain plant proteins can potentially lead to celiac disease (Dahal-Koirala *et al*., 2020). From an agricultural, climate and environmental perspective, non-digested protein from feed contributes significantly to N leaching into the environment. The European Commission has recognized the urgent need to cut down nutrient loss by at least 50% by 2030 under the Green Deal program (The European Commission, 2020). Efficient and non-inhibited degradation of plant-based feed protein is vitally important for reaching this goal.

Grains from cereals contain several anti-nutritional factors interacting with enzymes important for the degradation of phytin (phytase), non-starch polysaccharides (xylanase) and proteases (Bekalu *et al*., 2017; Dornez *et al*., 2009; Nørgaard *et al*., 2019). While the effect of cereal xylanase inhibitor levels on animal feed digestibility is well known (Krogh Madsen *et al*., 2018), the impact of individual cereal protease inhibitors is largely undescribed. The collection of protease inhibitors is huge, and each can potentially inhibit proteases of importance in foods and feed. The inhibition of proteases can potentially also affect celiac disease management because epitope peptides are not degraded, resulting in an immune response (Dahal-Koirala *et al*., 2020). *In planta*, endogenous grain protease inhibitors have a main function in the timing of protein hydrolysis prior to and during germination, as well as functioning as a defence against fungal- and pest proteases (Abd El-latif, 2014; Pekkarinen *et al*., 2003; Pekkarinen and Jones, 2002). The latter is urgently important to keep in mind and evaluate in plants with modulated protease inhibitor levels. However, protease inhibitors are abundant in the grain, and the addition of exogenous proteases to feed and food exceeding the level of inhibitors is not considered a viable solution. Moreover, varying levels and composition of protease inhibitors between cultivars and batches of cereals affect protease activity differently, potentially leading to different protein digestibility levels in e.g. feed. In line with this, significant cultivar differences in wheat grain xylanase inhibitor levels have already proved to affect the growth and feed rate in poultry feeding studies (Krogh Madsen *et al*., 2018).

The present study provides insight into an array of six barley grain protease inhibitors that alone or in combinations may influence the digestion of barley grain proteins and the world's most important source of plant protein, soy protein (Pope *et al*., 2024; Stein *et al*., 2008; Zhang *et al*., 2013). In addition to being important food and feed proteins, the abundant

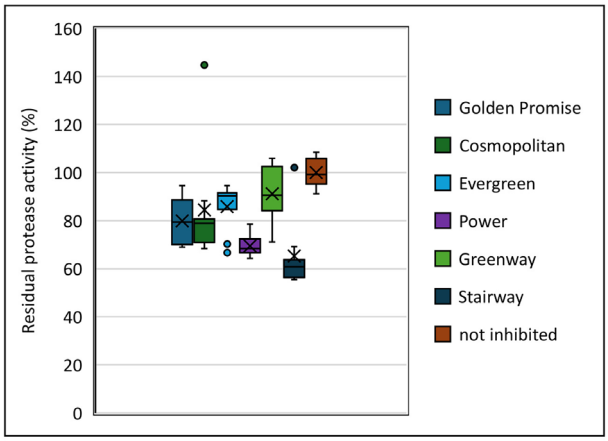

Figure 1 Box plots inclusive medians. Dots represent outliers and (×) is the mean value. Residual Ronozyme ProAct activity assay on elite barley cultivars and Golden Promise with 40 μL (100% grain protein fraction). The proteolysis of the AZCL-Casein substrate is measured at 600 nm.

soybean storage proteins glycinin and β-conglycinin are regarded as allergenic proteins and can cause hypersensitivity (Krishnan *et al*., 2009; Wang *et al*., 2014, 2023).

The implication of barley grain serine protease inhibitors on externally added proteases was studied. These included chymotrypsin inhibitors (CI-1A, CI-1B and CI2), barley α-amylase/subtilisin inhibitor (BASI) a bifunctional inhibitor belonging to the Kunitz-type trypsin inhibitor family (Nielsen *et al*., 2004), Bowman-Birk trypsin inhibitor (BBI) and Serpin Z4. The most abundant protease inhibitors in the barley grain are the serpin Z proteins. There are three Z proteins present in the endosperm of barley, Zx, Z4 and Z7, with Z4 being the most abundant of the three and Z4 and Z7 accounting for up to 5% of the grain protein (Evans and Hejgaard, 1999). The serpins have a dual function in the grain as both serine protease inhibitors and as storage proteins, accounting for a large portion of the grain lysine (Hejgaard *et al*., 1985). Additionally, Z proteins have been identified as a beer component improving beer foaming quality (Evans and Hejgaard, 1999).

As a potentially novel approach to increase the nutritional value of plant protein, the levels of the barley protease inhibitors were reduced by simplex and multiplex CRISPR/Cas9 mutagenesis. Finally, selected mutants were tested for their effect on the degradation of recombinant barley hordein storage proteins B and C and native soy protein.

Results

Protease inhibition in model and modern barley cultivars

Golden Promise is a model barley cultivar used primarily because of its favourable tissue culture properties and is not widely grown as an agricultural crop (Bekalu *et al*., 2023). To put our study in the context of today's agriculture, we therefore tested modern barley cultivars for their inhibition of commercial Ronozyme ProAct protease with 100% grain protein extract (Figure 1). Many of the cultivars tested have a similar effect on residual protease activity as Golden Promise (70%–90%), but some differ. Greenway had the highest residual protease activity with 91% of all the cultivars tested. At the other end of the scale, Stairway allowed only 65% residual activity of the protease.

Generation of CRISPR/Cas9 loss of function mutants

Six protease inhibitor genes (*CI-1A*, *CI-1B*, *CI2*, *Serpin-Z4*, *BBI* and *BASI*) expressed in the mature barley grain were identified for the cultivar Morex in the Ensembl barley genome (https://plants.ensembl.org/Hordeum_vulgare/Info/Index). Protospacers for the knockout of these genes were designed for the CRISPR/Cas9 constructs (Table 1). As the transformation target was the barley cultivar cv Golden Promise, the selected protospacer sequence of each gene was verified by sequencing the corresponding target regions in Golden Promise. The *CI-1A* gene sequence in Stairway was also sequence-verified as being the same as in Golden Promise. Golden Promise simplex and multiplex knockout mutant transformants were generated, and mutations were identified by sequencing (Figure 2). Transformation efficiency and regenerated plants zygosity can be seen in Tables S1 and S2. Primary mutants with homozygous or bi-allelic out-of-frame mutations were selected for further studies when possible (Figure 2). Three such primary mutants could be selected for each of the *Serpin-Z4*, *BBI*, *CI-1A* and *CI2* genes and the double knockout of genes *CI-1B* and *CI-1A*. For the *BASI* gene knockout, two such lines could be selected, and only one such primary mutant was generated with the triple chymotrypsin inhibitor knockout *CI-1B/CI-1A/CI2*. Predicted amino acid translations of the mutations are shown in Figure S1. Most mutants show a non-sense frameshift resulting in a premature stop codon. The exceptions are the bbi mutant #3 and bbi mutant #14 allele 2, showing non-sense translation but extended sequence before a stop codon. The *CI-1B* and *CI-1A* double mutant plant number 3 has a three-base deletion resulting in a single amino acid deletion in ci-1a #3 allele 2 (Figure 2 and Figure S1). Three regenerated Golden Promise plants with wild-type genotype were also included as controls.

Table 1 Protease inhibitors selected for CRISPR/Cas9 mutation

Gene name	Accession nr.	Protospacer sequence (5′-3′)
Chymotrypsin inhibitor 1A (CI-1A)	HORVU.MOREX.r3.1HG0012580	GAAGAACATGAGTTCCATGG**AGG**
Chymotrypsin inhibitor 1B (CI-1B)	HORVU.MOREX.r3.1HG0012570	**CCT**CCATGGAACGCATGTTCTTC
Chymotrypsin inhibitor 2 (CI2)	HORVU.MOREX.r3.1HG0012640	**CCG**TCACAACCTGAAGACAGAG
Serpin-Z4	HORVU.MOREX.r3.4HG0342810	**CCG**AGCGTGCTGCCGGCAATGTC
Bowman-Birk trypsin inhibitor (BBI)	HORVU.MOREX.r3.3HG0222410	GTGGAAGTGCTGCGACCAGG**CGG**
Barley α-amylase/subtilisin inh. (BASI)	HORVU.MOREX.r3.2HG0183360	**CCG**ATCCGCCGCCGGTGCACGAC

Their accession number and protospacer sequence. PAM site as bolded.

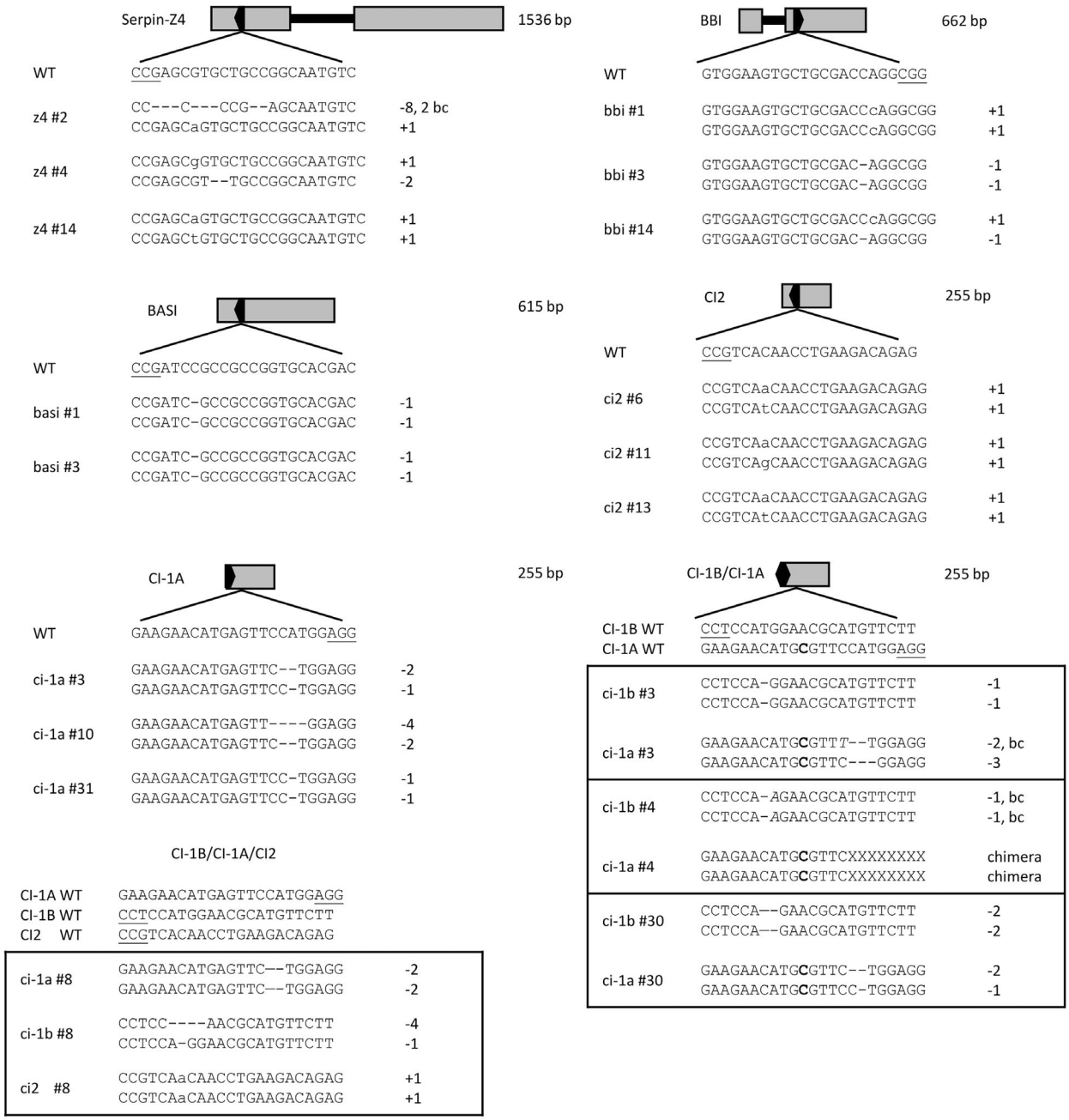

Figure 2 Schematic figures of protease inhibitors and mutations in Golden Promise. The wild type sequence of the protospacer sequence (WT) and both alleles of the mutant sequences selected for further analysis. Grey box, exons. Black line, introns. Black arrow, location of protospacer sequence in the gene and its orientation. The triple and double mutant lines are divided in the black boxes. Bc, base change.

Two of the chymotrypsin inhibitors (*CI-1A* and *CI-1B*) have a high gene sequence homology of 93.1% (Figure S2a). As a result of this, the designed protospacers for these two genes have a single nucleotide difference (A/C) at the tenth nucleotide from the PAM site (Figure S2b). This SNP is at the border of what is designated as the seed region of the protospacer, normally defined as being between the 8th and 10th nucleotides proximal to the PAM site (Soyars *et al.*, 2018). By using the border-lining SNP, we sought to generate both single and double mutants using a single guide with the protospacer designed for the *CI-1A* gene or the protospacer designed for the *CI-1B* gene in a simplex system (Table 1). The CRISPR/Cas9 construct with the protospacer designed for the *CI-1A* only induced mutations in the *CI-1A* gene but did not induce any mutation in the *CI-1B* target gene, being wild type in all plants (Figure S2c). On the other hand, the CRISPR/Cas9 construct with the protospacer designed for the *CI-1B* gene induced mutations in both the *CI-1B* and the *CI-1A* gene in all transformants screened (Figure S2c). We therefore managed to obtain single knockout mutants of chymotrypsin inhibitor 1A (ci-1a) and double knockout mutants of chymotrypsin inhibitors 1A and 1B (ci-1b/1a).

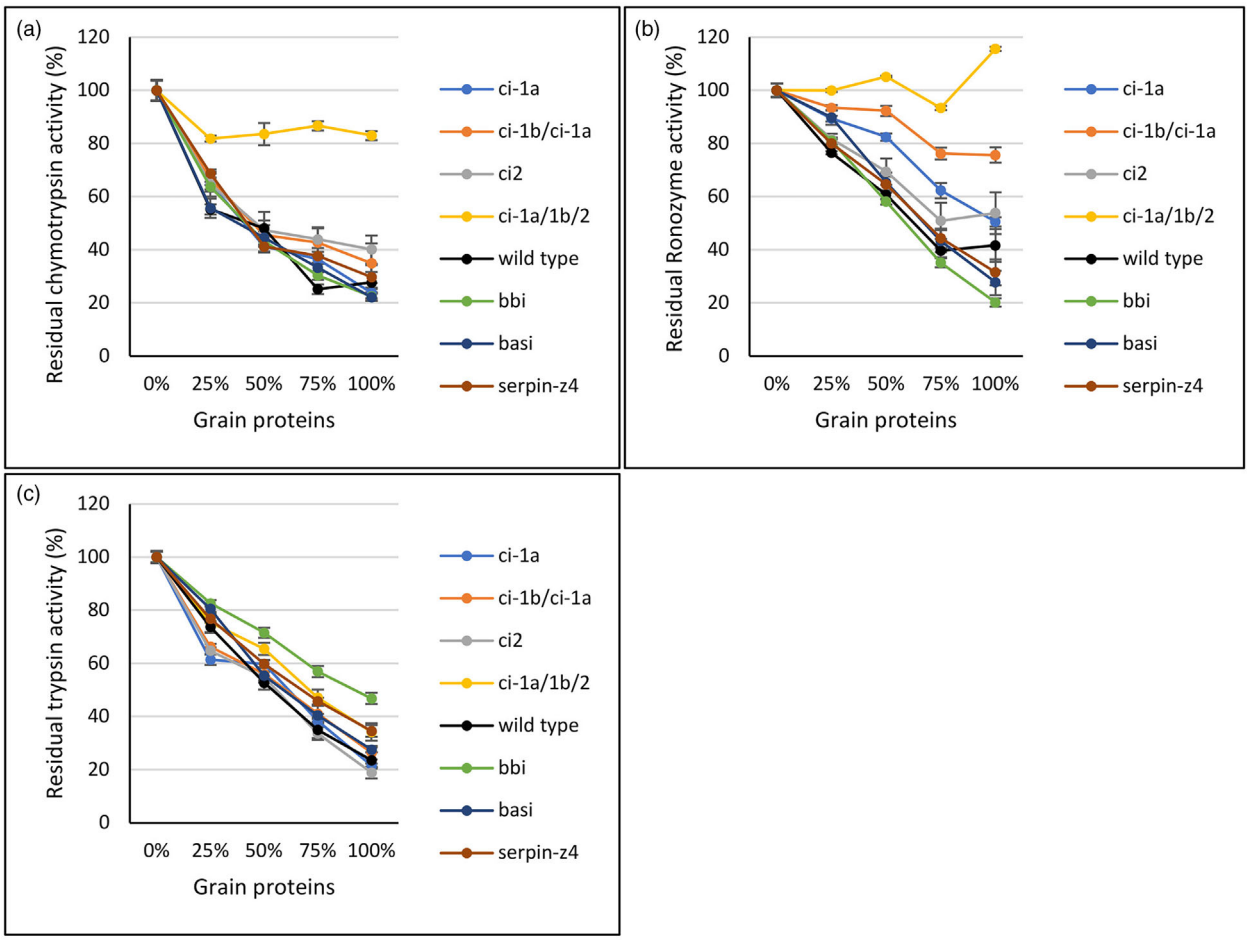

Figure 3 Residual protease activity assay. Extracted water-soluble grain protein fractions from mutants or wild type mature grains are mixed with a protease to measure the inhibitory effect of protease inhibitors in the grain. Samples from each plant are run in a technical triplicate. The wild type ci-1b/1a double mutant and all single mutants have three biological replicates (n = 9), except BASI with two mutant plants (n = 6). Three separate protein extractions from the ci-1a/1b/2 triple mutant were used (n = 9). The proteolysis of the AZCL-Casein substrate is measured at 600 nm. (a) Residual protease activity of α-chymotrypsin. (b) Residual protease activity of Ronozyme ProAct. (c) Residual protease activity of trypsin.

The three genes *CI-1A*, *CI-1B and CI2* are chymotrypsin inhibitors, belonging to the Potato type I inhibitor family. Their identical molecular function as serine-type endopeptidase inhibitors (gene ontology, GO: 0004867) results in an overlap of inhibitory functions, as described previously (Greagg *et al.*, 1994). To investigate the additive effect of these three CI genes, we generated a multiplex triple chymotrypsin inhibitor knockout (ci-1a/1b/2).

Effect of mutations in barley on external protease activity

Mutant and wild type barley grain proteins were assayed for their inhibition of the three different serine proteases: α-chymotrypsin (C4129, Sigma-Aldrich), trypsin (T8003, Sigma-Aldrich) and the commercial feed additive protease Ronozyme ProAct, a subtilisin with mainly chymotrypsin activity (Novozymes, Denmark). Mature grains were milled and different amounts of water-soluble protein extracts from the flour (0%, 25%, 50%, 75% and 100%) were mixed with the proteases. These extracts will be referred to as grain proteins. The residual activity of α-chymotrypsin showed that wild type grain protein at 25% reduced the protease activity to 55% (Figure 3a). The activity decreased further to 25% and 28% when 75% and 100% grain proteins were added, respectively. With the additions of 75% wild type grain protein, all mutants performed significantly better than the Golden Promise wild type, except for the bbi mutants. At 100% grain protein, only the ci-1a/1b/2 triple mutant performed significantly better than the wild type. The residual protease activity of the ci-1a/1b/2 triple mutant was at all grain protein fractions significantly higher in the mutated line than in the wild type. The residual activity of the ci-1a/1b/2 triple mutant stayed between 81.8% and 86.7%, regardless of the amount of grain protein added to the reaction. At 25% grain protein, the single ci-1a mutants and ci-1b/1a double mutants showed with 65% and 67% residual activity, respectively, a significantly increased residual protease activity as compared to the wild type. The ci2 mutants had the highest residual activity of the single mutants, ranging from 65% activity with 25% grain protein to 40% with 100% grain protein (Figure 3a).

Next, the residual activity of Ronozyme ProAct protease was measured (Figure 3b). The inhibition of Ronozyme ProAct by the wild type grain protein extract was less than the inhibition of α-chymotrypsin, ranging from 76% activity at 25% grain protein and having the lowest activity at 75% grain protein with 40%

activity. Grain proteins from bbi and basi mutants both had a significantly higher residual activity than the wild type when 25% grain protein was added, displaying a residual activity of 81% and 90%, respectively. However, at 100% grain protein, the bbi mutant had significantly lower activity than the wild type, with only 20% residual activity, while the basi mutant showed 28% residual activity. The knockout of chymotrypsin inhibitors had a great effect on the residual activity of Ronozyme ProAct (Figure 3b). With 25% grain protein, all four knockout combinations had a significantly positive effect on Ronozyme ProAct activity. The ci2 mutant displayed a minor increase with 82% residual activity. Ci-1a, ci-1b/1a double mutant, and ci-1a/1b/2 triple mutant had 89%, 93% and 100% residual activity, respectively. With 50%, 75% and 100% grain protein, the ci2 mutant still had the lowest residual activity at 69%, 51% and 54%, respectively. The ci-1a mutant showed significantly higher activity with 50% and 75% grain protein added (82% and 62% activity left). At 100% grain protein, the Ronozyme ProAct activity was reduced to 51%. The ci-1b/1a double mutant showed a strong positive effect on the residual activity compared to the wild type at all levels of added grain protein, 92%, 76% and 75.7% residual activity when 50%, 75% and 100% grain proteins were added, respectively. Again, the ci-1a/1b/2 triple mutant showed the highest effect on the residual protease activity (Figure 3a,b). Interestingly, at both 50% and 100% albumin, the activity exceeded the starting level of pure Ronozyme ProAct protease (0% grain protein, 100% activity). Surprisingly, the highest activity (116%) was found with the highest amount of grain protein added. Serpin-Z4 showed almost the same residual activity as the wild type at all concentrations of grain protein (Figure 3b).

The third protease measured for its residual activity with grain protein fractions was bovine trypsin (Figure 3c). As expected, there was less effect of the knockout of chymotrypsin inhibitors. Indeed, at 25% grain protein, ci-1a, ci-1b/1a and ci2 had lower activity than the wild type (61%, 66%, 65% and 74%, residual activity detected respectively). The residual activity level of the ci-1a/1b/2 triple mutant was like the wild type (75%) with 25% grain protein. At 50%, 75% and 100% grain protein, ci-1a/1b/2 showed higher residual activity than the wild type with 65%, 47% and 34% residual trypsin activity in the triple mutant as compared to 53%, 35% and 23% in the wild type. The basi mutant retained a higher residual activity at 25% grain protein, but not when adding more grain protein. The serpin-z4 mutant performed better at 50% grain protein with residual trypsin activity levels at 60%, 46% and 35% with 50%, 75% and 100% grain proteins, respectively. The bbi knockout mutant displayed the highest effect on the residual activity of trypsin, being significantly higher than the wild type at all grain protein fractions. The residual trypsin activity was 83% at 25% grain protein, 71% at 50% grain protein and 47% at 100% grain protein.

Native protein degradation

The triple chymotrypsin mutant ci-1a/1b/2 was the best performing mutant in the residual protease assay using α-chymotrypsin or Ronozyme ProAct (Figure 4a,b). This mutant was therefore selected for a recombinant protein degradation assay of storage barley proteins. The storage proteins B- and C-hordein were expressed in *E. coli* and purified. The recombinant storage proteins were mixed with the water-soluble grain protein fraction of the triple mutant or wild type. Controls having no protease and

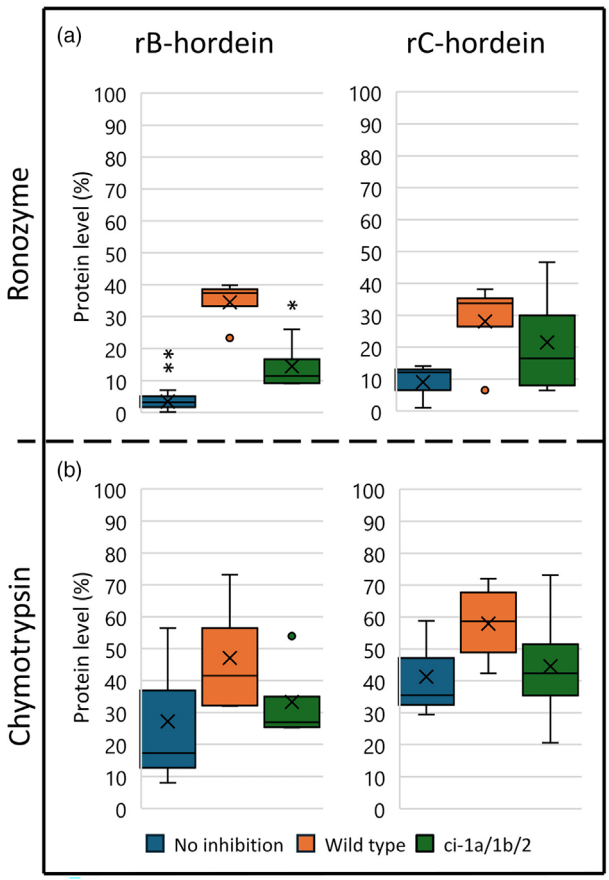

Figure 4 Box plots inclusive medians of remaining substrate protein level (%). The band intensities within an area on SDS-PAGE gels were estimated. (×) is the mean value and the dots are outliers. Protein levels were calculated from the protein level of a control with only substrate (20 μg storage protein) given as 100% protein level. The Golden Promise ci-1a/1b/2 triple mutant and three wild type regenerated plants water-soluble grain protein fractions were incubated with either recombinant B- or C-hordein and tested for their inhibition of Ronozyme ProAct and α-chymotrypsin (a and b). A mix of three independent grain protein extractions from the triple mutant was pooled before incubation and loading on the gel. The same was done for the three wild types. Four independent runs were made and used for protein level calculations ($n = 4$). Samples without any grain proteins were included for all experiments (No inhibition, $n = 3$ in all experiments). Significant differences were calculated by two-tailed Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

grain protein (pure hordein) were used for calculating the relative degradation of hordeins, measured as the adjusted volume of band intensities in per cent (protein level, %). A control with only protease and hordein (no inhibition) was included to see how much recombinant hordein would be degraded without inhibition (Figure 4a,b). With Ronozyme ProAct protease and the non-inhibited control, almost all the rB- and rC-hordein substrates were degraded (Figure 4a). The wild type grain inhibited the degradation of rB-hordein using Ronozyme ProAct with a mean residual substrate protein level of 34.5%. Both the ci-1a/1b/2 triple mutant and no inhibition samples inhibited degradation significantly less than the wild type, with substrate protein levels at 14.5% and 3.4%, respectively (Figure 4a). With the rC-hordein Ronozyme ProAct degradation (Figure 4a) and the degradation of

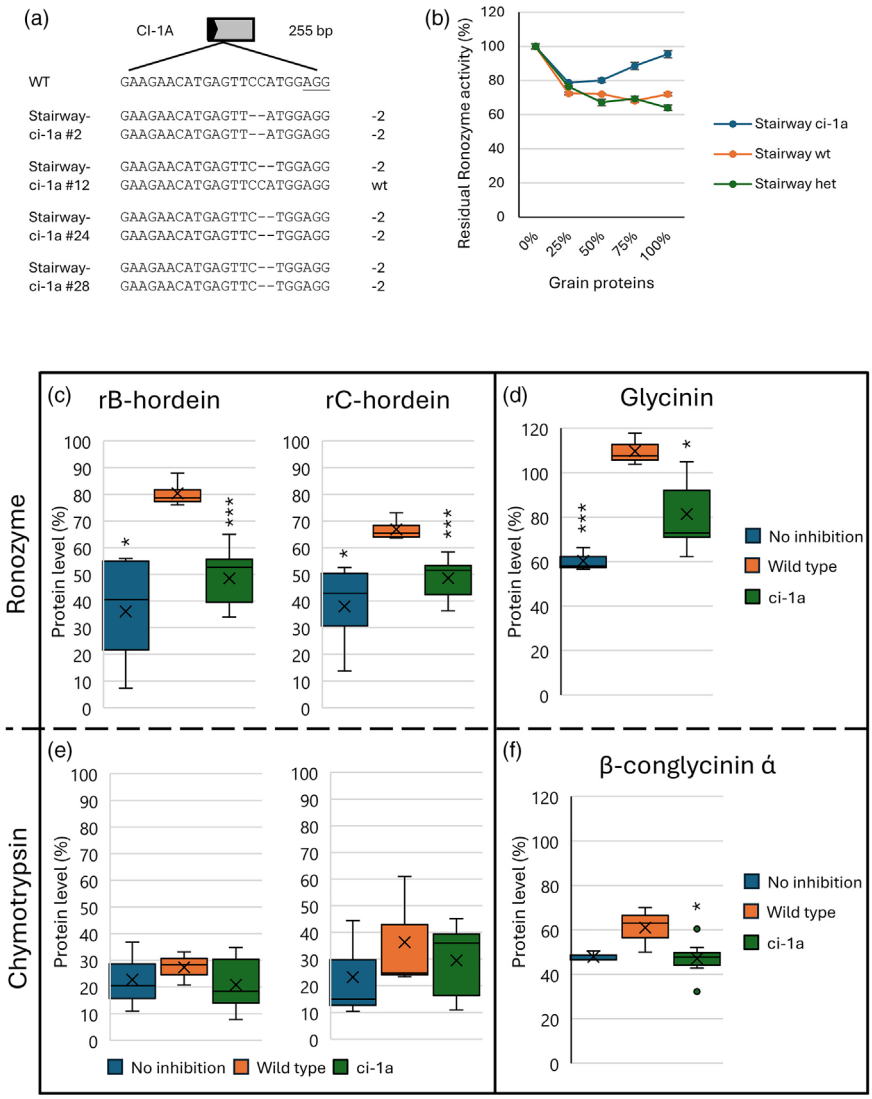

Figure 5 Stairway cultivar mutants. (a) Schematic figure of CI-1A protease inhibitor mutations in T_1 generation Stairway ci-1a mutants. The described lines are used in all subsequent experiments. Grey box, exons. Black arrow, location of protospacer sequence in the gene and its orientation. (b) Residual protease activity of Ronozyme in the T_1 generation of Stairway mutants, Stairway wild type, and Stairway heterozygous mutants. The proteolysis of the AZCL-Casein substrate is measured at 600 nm. Protease inhibition on 40 μg recombinant rB- or rC-hordein was tested with the water-soluble grain proteins from the Stairway ci-1a mutants and the wild type and run on SDS-PAGE gels (c, Ronozyme ProAct and e, α-chymotrypsin). The three T_1 mutant siblings were run independently three times and the protein levels were pooled (n = 9). The wild type T_1 sibling was run three times (n = 3). Inhibition of Ronozyme ProAct and α-chymotrypsin by Stairway ci-1a mutants and wild type grain proteins on degradation of soy storage proteins glycinin (d) and β-conglycinin ά (f). Samples were run like the hordein experiment with n = 9 for the Stairway ci-1a mutants and n = 3 for the wild type. Samples without any grain proteins were included for all experiments (No inhibition, n = 3 in all experiments). Significant differences were calculated by two-tailed Student's t-test. *P < 0.05, ***P < 0.001.

both recombinant proteins with α-chymotrypsin (Figure 3b), there is a tendency for the wild type samples to have a higher inhibition. This is the case for both the non-inhibited sample and ci-1a/1b/2 triple mutant samples.

CRISPR/Cas9 mutagenesis of the modern barley cultivar Stairway

Having the highest potential for improvement (Figure 1), the commercial barley cultivar Stairway was selected for targeted mutagenesis of protease inhibitors. Stairway was mutated in the *CI-1A* gene through *Agrobacterium*-mediated transformation of the ovule. The procedure, including embryo development from the zygote within the ovule and the subsequent plant regeneration from the embryo, was performed without a selection agent in the culture media used. One mutant plant was regenerated (Figure S3a). The sequencing of the target site in the *CI-1A* gene of the T_0 mutant revealed a bi-allelic mutation at the target site, but sequencing of the T_1 progeny showed that it was chimeric, with wild type, heterozygous and homozygous mutant offspring (Figure S3b,c). In the T_1 offspring, we identified one wild type plant, three homozygous ci-1a mutant plants and two heterozygous plants (Figure 5a). One of these (number 11) could only be verified in the PCR/RE assay, while no sequence could be obtained. The amino acid sequence of the mutations shows

a premature stop codon shortly after the mutations (Figure S3d). These were tested for the residual protease activity assay using Ronozyme ProAct, because this protease showed the highest variation in Golden Promise (Figure 3b). The wild type and heterozygous mutant plants show very limited variation in the residual protease activity (Figure 5b). On the other hand, the Stairway ci-a1 homozygous mutants showed significantly increased residual activity from 50% grain protein levels and up. Interestingly, residual protease activity increased from approximately 80%–96% when 50%–100% grain protein was added. The wild type plant had 72% residual activity with 100% grain protein (Figure 5b).

Next, degradation of recombinant barley storage proteins rB- and rC-hordein with either Ronozyme ProAct or α-chymotrypsin proteases was studied (Figure 5c,e). Grain protein fractions were added from wild type or the Stairway ci-1a homozygous T_1 mutants (ci-1a mutants). The Ronozyme ProAct protease degraded significantly more rB- and rC-hordein in the ci-1a mutant lines compared to the wild type line (Figure 5c).

A-Chymotrypsin was able to degrade most of both rB- and rC-hordein in all samples. The highest remaining substrate protein levels were in the wild type with a mean of 27.4% and 36.6%, respectively (Figure 5e). To compare, the ci-1a mutants had a mean remaining substrate protein level of rB- and rC-hordein at 20.8% and 29.5%, respectively.

Soy storage protein degradation

One of the most important plant protein sources is soy proteins. The two most abundant storage proteins in soy are the water-soluble glycinin (11S glycinin) and β-conglycinin (7S glycinin) accounting for 70%–80% of total protein content in the mature grain (Singh *et al*., 2015). The Stairway ci-1a mutants were studied for their potential to stimulate improved soy protein degradation (Figure 5d,f). The substrate protein level (%) from the SDS-PAGE gel of the degradation assay was estimated, relative to the pure soy samples (100%). The degradation of glycinin by Ronozyme ProAct was significantly higher in the no inhibition sample and ci-1a mutants sample compared to the wild type (Figure 5d). It appears that the grain protein fraction has protein at the same size as glycinin (~28 kDa), giving a protein level of 109.7%, which is higher than the pure soy sample. Degradation of the β-conglycinin bands around 72 kDa (type ά) by α-chymotrypsin was significantly higher in the ci-1a mutants than the wild type (Figure 5f). The remaining substrate protein level was the same in the ci-1a mutants as for the no inhibition sample (47.1% and 47.8%, respectively).

In order to determine if there were any negative phenotypical impacts of the mutants, we evaluated the agronomic traits spike length, spike number and TKW in the Golden Promise ci-1a/1b/2 triple mutant and the Stairway ci-1a mutant. No significant differences were detected in any of the parameters (Figure S4a). Additionally, the spike morphology remained unchanged for all studied mutants (Figure S4b).

Discussion

Grain protease inhibitors represent significant anti-nutritional factors, reducing the nutritional value of grain protein in food and feed. Inefficient protein digestibility has significant implications for the health value of a diet and on how much non-digested nitrogen is secreted into the environment via the manure. Our study aimed to identify important protease inhibitor genes that function as negative regulators of the proteolysis of the major storage proteins from barley and soybean. By using simplex and multiplex CRISPR/Cas9 mutagenesis, we aimed to increase the digestibility of barley grain proteins and soy protein by removing serine protease inhibitors in the grain (Adeola and Cowieson, 2011; Canibe and Jensen, 2003; Christensen *et al*., 2014; Hejgaard and Boisen, 1980; Holme *et al*., 2012a; Nørgaard *et al*., 2019). Our research included studies in the model barley cultivar Golden Promise and in modern commercial barley cultivars. Six protease inhibitor candidates were selected and single knockout mutants were obtained for Serpin-Z4, BASI, BBI, CI-1A and CI2. In addition, CI-1A/CI-1B double mutants and a CI-1A/CI-1B/CI2 triple mutant plant were obtained by multiplex CRISPR/Cas9 (Figure 2). Due to high sequence homology between the *CI-1A* and *CI-1B* genes and only a single SNP in the protospacer sequences, we did not obtain a single CI-1B mutant. Interestingly, the mutation rate differed between the two guides at their alternative target site, with the *CI-1B* guide generating mutations in the *CI-1A* gene, while the *CI-1A* guide did not generate any mutations at the alternative *CI-1B* target site. Further studies are required to uncover the background for this difference.

With the exception of Golden Promise and a few other cultivars, barley is known to be recalcitrant in tissue culture, and hence also gene editing (Bekalu *et al*., 2023). However, Golden Promise is of only limited relevance for current agriculture, and we therefore decided to involve modern and relevant cultivars in our study. Five different modern barley cultivars and Golden Promise were tested *in vitro* for their inhibitory effect of the Ronozyme ProAct feed protease (Figure 1). The inhibition varied significantly between the cultivars. The Greenway cultivar caused the least inhibition, maintaining 91.1% protease activity, while the cultivar Stairway showed the highest inhibition, leaving only 65.2% activity. The varying levels of protease inhibition between cultivars are in line with cultivar differences seen for inhibition of the nutritionally important enzymes xylanase and phytase (Bekalu *et al*., 2017; Krogh Madsen *et al*., 2018).

By using the barley ovule as the starting explant and no selective agent in the media used during the culture of the *Agrobacterium* infected tissue, a mutation in the *CI-1A* gene was introduced by CRISPR/Cas9 in Stairway, the cultivar with the highest endogenous protease inhibition. Barley ovules have previously been demonstrated as a source for cultivar-independent explants for genetic transformation (Holme *et al*., 2008). However, the current study is the first demonstration of the barley ovule as an explant for gene editing in barley. Moreover, the gene editing was done in the commercial cultivar Stairway, which in our lab has been impossible to approach for transformation and gene editing via the standard procedure using immature embryos as the starting material. The whole procedure was carried out without the use of a selective agent. To our knowledge, this is the first example of gene editing in commercial barley not involving a selective agent.

From the T_0 chimeric Stairway mutant, we selected three homozygous mutants, two heterozygous mutants and one wild type T_1 progeny. The level of residual activity of Ronozyme ProAct after assaying with grain water-soluble protein from the homozygous ci-1a mutants was significantly increased when compared to the heterozygous and wild type plants. The activity even increased with increased grain protein (Figure 5b). A similar tendency was observed for the ci-1a/1b/2 triple mutant (Figure 3b), but the mechanism behind this needs to be studied

in detail. The single gene knockout of *CI-1A* in Stairway and Golden Promise had different effects on the residual activity of Ronozyme ProAct. The Stairway ci-1a mutants had a 95.5% residual activity compared to 50.5% residual activity in the ci-1a Golden Promise mutants when assayed with 100% grain protein. The result demonstrates how the level of a single protease inhibitor can vary between cultivars. The current study represents a conceptual study but does also indicate that the level of individual protease inhibitor contributions in a cultivar must be taken into account in the planning of a breeding strategy supporting a better proteolysis of storage proteins.

In Golden Promise, there is a clear increase in residual activity of α-chymotrypsin and Ronozyme ProAct protease when knocking out all three chymotrypsin inhibitor genes compared to knocking out only one or two genes (Figure 3b). The ci-1a/1b/2 triple mutant shows an activity higher than 100% when assayed with 100% grain protein extracts, which is more than the protease without added grain protein. One explanation could be that endogenous proteases are present in the crude water-soluble fraction of barley grain proteins, affecting the degradation of the substrate.

As expected, as a trypsin inhibitor, the *BBI* knockout had the highest effect on the level of residual activity of trypsin after mixing with grain protein. The *bbi* mutant showed the highest increase in protease activity of all mutants from 25% to 100% added grain protein. Knockout of the *Serpin-Z4* gene encoding the very abundant Serpin-Z4 protein also showed a higher residual trypsin activity from 50% grain protein and above (Figure 3c).

In barley, the most abundant grain storage protein is B-hordein, accounting for 70%–90% of the total hordeins, followed by C-hordeins ranging from 10% to 30% of the total hordeins (Shewry *et al*., 1985; Tanner *et al*., 2019). In this study, improved degradation of recombinant barley hordeins by Ronozyme ProAct or α-chymotrypsin was seen in the Golden Promise chymotrypsin triple mutant. The recombinant rB-hordein was significantly more degraded by Ronozyme ProAct in the presence of grain protein extracts from ci-1a/1b/2 triple mutant than extracts from the wild type (Figure 4a). Also, the extracts from the Stairway ci-1a mutants led to significantly improved digestibility by Ronozyme ProAct of recombinant rB and rC hordeins as compared to extracts from the wild type (Figure 5c). The Stairway ci-1a mutants were also studied for their effect on the degradation of soybean storage proteins (Zhang *et al*., 2013). Also, here we found that the mutants performed better, with higher degradation by Ronozyme ProAct or α-chymotrypsin of glycinin and β-conglycinin compared to the wild type (Figure 5d,f). Our results provide the first insight into a novel way of mutating barley that potentially can lead to increased digestibility of important storage proteins from barley and soybean and suggest that future efforts could be directed towards understanding how these changes could be utilized in the context of food and feed.

In conclusion, the findings of this study could prove to have a positive nutritional impact on barley used as feed or food and perhaps also on malting quality. It could also result in less or better utilization of protein sources, i.e. soy or protease additives in feed.

Experimental procedures

Vector construction

The vector system used for CRISPR/Cas9-induced mutations relies on the destination vector pANIC6A (Mann *et al*., 2012). In this system, two entry vectors are used: the pJG85 (addgene #89281) entry vector for insertion of the specific synthetic guide sequences (sgRNAs) and pJG80 (addgene #89282) containing the Cas9 gene, codon optimized for wheat (Gil-Humanes *et al*., 2017). The ligation of sgRNA sequences into pJG85 and subsequent LR clonase reaction with the two entry vectors and the destination vector to assemble the final expression vector has previously been described (Panting *et al*., 2021). The sgRNA oligo sequences for all six genes can be seen on Table S3. Linearization of the entry vector using Esp3I leaves a 4 bp overhang as well as removes the first base in the scaffold RNA sequence that needs to be reconstituted in the sgRNA oligos.

The final expression vectors were transformed into *Agrobacterium tumefaciens* strain AGL0 using the freeze/thaw method. The transformed *Agrobacterium* cells were grown at 28 °C in solid or liquid LB media containing 25 μg/mL rifampicin and 50 μg/mL kanamycin antibiotics for selection.

Introducing CRISPR/Cas9 constructs in barley immature embryos and zygotes

The donor plants (cv. Golden promise and cv. Stairway) used for transformation were grown in a growth chamber with a 16 h light period with 350 μ E m^{-2} s^{-1} and 15 and 10 °C day and night temperatures, respectively. Stable transformation of cv. Golden Promise was done by *Agrobacterium*-mediated transformation of immature embryos. Twelve- to fourteen-day-old embryos were isolated and transformed as previously described (Holme *et al*., 2017). Briefly, the axis was cut away from the scutellum using a scalpel dipped in an overnight *Agrobacterium* culture containing no antibiotics. When generating the triple chymotrypsin inhibitor mutant, the overnight *Agrobacterium* cultures for each of the three genes were mixed 1:1:1 just prior to immature embryo transformation. The selection agent hygromycin was used at a concentration of 50 mg/L in all media except the co-cultivation medium, which contained no selection agent.

Agrobacterium-mediated transformation of cv. Stairway used the zygote within the ovules as the transformation target (Holme *et al*., 2008, 2012b). Hand-pollination of emasculated spikes was performed 1 h before ovule isolation to ensure that the zygote within the ovules was just formed. Isolated ovules were subsequently infected with an overnight *Agrobacterium* culture containing no antibiotics. A fine needle (0.4 mm × 19 mm) dipped in the overnight culture was used to puncture the ovule embryo sac and release *Agrobacterium* into the sac containing the zygote. Subsequently, a plant was regenerated as described (Holme *et al*., 2008). The entire procedure was performed without a selection agent in any of the culture media.

Mutant screening of genotyping

Approximately 10 cm young leaf pieces were cut and immediately frozen in liquid nitrogen in 2 mL tubes containing two 2 mm glass beads. The frozen leaves were crushed in a FastPrep-24 5G homogenizer (MP Biomedicals) at speed 6 for 10 s. DNA was extracted using the phenol/chloroform method.

Regenerated plants were initially screened by amplifying the hygromycin gene (Table S4), using Herculase II Fusion DNA polymerase (Agilent).

Hygromycin-positive plants were genotyped by amplifying the target region corresponding to the target in the construct also using Herculase II Fusion DNA polymerase (Agilent). The PCR products were either sequenced using the PCR amplification primers or TOPO cloned (Zero Blunt™ TOPO™, Invitrogen™), with

6–8 clones sequenced. Primers used for the different CRISPR/Cas9 targets and the PCR product sizes can be seen in Table S4.

Protease inhibition assay

The protease inhibition assay was adapted from (Nørgaard *et al.*, 2019). The inhibition effect of barley grain extracted proteins was measured for three different proteases, bovine trypsin (T8003, Sigma-Aldrich), bovine α-chymotrypsin (C4129, Sigma-Aldrich) and Ronozyme ProAct (Novozymes). Samples from all lines described in Figure 1 were used in the experiment. Each line was run in technical triplicates. The crude water-soluble albumin protein fraction (grain protein) of mature barley grains was extracted by mixing 250 mg barley flour with 2 mL of 0.1 M acetate, pH 5, as extraction buffer. The samples were shaken for 1 h in a horizontal shaker and centrifuged at 3600 × ***g*** for 5 min at 4 °C. The water-soluble grain proteins in the supernatant were transferred to new 1.5 mL Eppendorf tubes and kept on ice until use or at 4 °C for short-term storage. Trypsin and α-chymotrypsin were dissolved in 0.1 M HCl (1 mg/mL) just prior to use, and Ronozyme ProAct was diluted 1:100 before use. The assay is based on the cleavage and release of the chromogenic compound (azurine) from its crosslink to casein (AZCL-casein, Protazyme AX, Megazyme, Ireland). The AZCL-casein substrate was prepared by dissolving two tablets in 10 mL of the reaction buffer, keeping the substrate on a magnetic stirrer all the time. The reaction buffer for Ronozyme ProAct was 0.1 M Tris/HCl, pH 8. The reaction buffer for trypsin and α-chymotrypsin was 0.1 M Tris/HCl, pH 7.8 with 10 mM $CaCl_2$.

The assay was performed with different amounts of soluble grain protein fraction, i.e., 40 μL (100%), 30 μL (75%), 20 μL (50%), 10 μL (25%) and 0 μL (0%). Extraction buffer was added to the 75%, 50% and 25% grain protein fractions for a total of 40 μL. The protease activity was also measured without any grain proteins (non-inhibited sample, 0%). A blank control with only buffers was also included. The grain protein fractions were pre-incubated in a 2 mL Eppendorf tube with 10 μL of protease for 5 min at room temperature. The samples were transferred to a 37 °C heating block, and 300 μL AZCL-casein buffered substrate was added. The reaction was incubated for 5 min with Ronozyme ProAct, 15 min with trypsin, and 30 min with α-chymotrypsin. The reaction was stopped by adding 150 μL 1.5 M HCl. The samples were centrifuged at 20 000 × ***g*** for 2 min, and 200 μL of supernatant was transferred in triplicate to a 96-well plate. The absorbance was measured at 600 nm using an Epoch microplate spectrophotometer (BioTek AG, Germany).

The pathlength-corrected absorbance was subtracted from the absorbance of the blank sample. The percentage of inhibition by the grain proteins was calculated by % protease inhibition = non-inhibited sample − sample/non-inhibited sample × 100. The remaining activity was calculated by subtracting this from 100%.

Barley storage protein degradation assay

Recombinant barley storage proteins C-hordein and B-hordein (rC-hordein and rB-hordein, respectively) were expressed and purified from *E. coli* shuffle T7 cells (New England Biolabs) as described previously (Rosenkilde *et al.*, 2014). Briefly, the expression of recombinant protein was induced by adding IPTG (final concentration of 0.5 mM) to an overnight grown 200 mL culture and left for 6 h at 25 °C. The cultures were centrifuged for 15 min at max speed. Retrieval of the recombinant protein was done using a sonication with an extraction buffer containing 0.1 M Tris/HCl pH 8.0 plus 1 mM PMSF with lysozyme. The sonicated homogenate was centrifuged at 4000 × ***g*** for 10 min, and the insoluble pellet was washed twice before further usage. The rC-hordein was purified from the soluble fraction after sonication and centrifugation using a 20 mL Ni/NTA column (Qiagen), following the manufacturer instructions. The rB-hordein was extracted in denaturing conditions with 8 M urea in the extraction buffer from the washed insoluble pellet. The urea-solubilized pellet was passed through a 20 mL Ni/NTA column (Qiagen), washed with a washing solution containing 20 mM imidazole, and eluted in 250 mM imidazole pH 8.0 containing 4 M urea. The concentration of purified recombinant hordeins was measured using the Bradford method (Bradford, 1976).

The grain protein fraction from the three wild type transformants was mixed 1:1:1, as well as the three separately extracted fractions from the ci-1a/1b/2 triple mutant. The storage protein degradation assay was done by mixing 60 μL reaction buffer (see above) with 1 μL α-chymotrypsin (1 mg/mL) or 1:100 dilution of Ronozyme ProAct and 6 μL of grain protein fraction from the ci-1a/1b/2 triple mutant or wild type. Samples were incubated for 5 min at room temperature and put on ice. Six μL recombinant hordein (20 μg total) was added, and the samples were incubated at 37 °C for 5 min. The reaction was stopped by putting the samples back on ice, adding 73 μL 2 × SDS-PAGE loading buffer, and boiling the samples for 5 min. The samples were spun down at 20 000 × ***g*** for 1 min and kept on ice until loading on the gel. Ten μL of each sample was run on Nupage 4%–12% Bis-Tris gels (Life-Technologies). A sample only containing the recombinant rB- or rC-hordein (pure hordein) was used to calculate a relative volume intensity, assuming each of those to be 100%. A control sample without grain protein (no inhibition) was included.

The degradation assay with the Stairway T_1 mutants was as described above, but with double the amount of recombinant hordeins in the reaction (40 μg total) and the Ronozyme ProAct protease was diluted 10 times more to 1:1000.

The relative volume intensities were calculated at the band of rB- or rC-hordein using the Volume tool in the Image Lab 6.1 software (Bio-Rad). The relative volume intensities (%) were calculated based on the pure hordein samples being 100%. This will be termed as protein level (%).

Soy protein extraction

Water-soluble soy proteins were extracted the same way as the water-soluble albumin fraction of barley flour (see above) using 0.1 M acetate, pH 5 extraction buffer. The degradation assay with the soy protein extract was done as described for the recombinant rB- and rC-hordein, but with 12 μL soy protein extract as substrate. The samples were run on SDS-PAGE gel and the relative volume intensities were calculated at the predominant glycinin band (~28 kDa) and two predominant β-conglycinin bands (~72 kDa) using the Volume tool in the Image Lab 6.1 software (Bio-Rad). The volume intensities were estimated from bands in an area of 7.7 mm^2 or 12.1 mm^2 for Glycinin and β-conglycinin, respectively. The relative volume intensity (%) was calculated from the adjusted volume intensities of samples, with a pure soy protein sample set to 100% and termed protein level (%).

Author contributions

MP: performed most of the experiments, data analysis and manuscript preparation. IBH: performed ovule transformation of

Stairway and helped with experimental setup and manuscript writing. GD: helped with identifying candidate genes and provided the recombinant hordeins. HB-P: developed the study concept, grant acquisition, manuscript writing and editing, and supervision.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

References

Abd El-latif, A.O. (2014) In vivo and in vitro inhibition of *Spodoptera littoralis* gut-serine protease by protease inhibitors isolated from maize and sorghum seeds. *Pestic. Biochem. Physiol.* **116**, 40–48.

Adeola, O. and Cowieson, A.J. (2011) BOARD-INVITED REVIEW: opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *J. Anim. Sci.* **89**, 3189–3218.

Bekalu, Z.E., Madsen, C.K., Dionisio, G. and Brinch-Pedersen, H. (2017) *Aspergillus ficuum* phytase activity is inhibited by cereal grain components. *PLoS One*, **12**, e0176838.

Bekalu, Z.E., Panting, M., Bæksted Holme, I. and Brinch-Pedersen, H. (2023) Opportunities and challenges of in vitro tissue culture systems in the era of crop genome editing. *Int. J. Mol. Sci.* **24**(15), 11920.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Canibe, N. and Jensen, B.B. (2003) Fermented and nonfermented liquid feed to growing pigs: effect on aspects of gastrointestinal ecology and growth performance. *J. Anim. Sci.* **81**, 2019–2031.

Christensen, J.B., Dionisio, G., Poulsen, H.D. and Brinch-Pedersen, H. (2014) Effect of pH and recombinant barley (*Hordeum vulgare* L.) endoprotease B2 on degradation of proteins in soaked barley. *J. Agric. Food Chem.* **62**, 8562–8570.

Dahal-Koirala, S., Neumann, R.S., Jahnsen, J., Lundin, K.E.A. and Sollid, L.M. (2020) On the immune response to barley in celiac disease: biased and public T-cell receptor usage to a barley unique and immunodominant gluten epitope. *Eur. J. Immunol.* **50**, 256–269.

Dornez, E., Gebruers, K., Delcour, J.A. and Courtin, C.M. (2009) Grain-associated xylanases: occurrence, variability, and implications for cereal processing. *Trends Food Sci. Technol.* **20**, 495–510.

Evans, D.E. and Hejgaard, J. (1999) The impact of malt derived proteins on beer foam quality. Part I. The effect of germination and kilning on the level of protein Z4, protein Z7 and LTP1. *J. Inst. Brew.*, **105**, 159–170.

Gil-Humanes, J., Wang, Y., Liang, Z., Shan, Q., Ozuna, C.V., Sánchez-León, S., Baltes, N.J. *et al*. (2017) High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. *Plant J.* **89**, 1251–1262.

Greagg, M.A., Brauer, A.B.E. and Leatherbarrow, R.J. (1994) Expression and kinetic characterization of barley chymotrypsin inhibitors 1a and 1b. *Biochim. Biophys. Acta*, **1222**, 179–186.

Hejgaard, J. and Boisen, S. (1980) High-lysine proteins in hiproly barley breeding: identification, nutritional significance and new screening methods. *Hereditas*, **93**, 311–320.

Hejgaard, J., Rasmussen, S.K., Brandt, A. and Svendsen, I. (1985) Sequence homology between barley endosperm protein Z and protease inhibitors of the α1-antitrypsin family. *FEBS Lett.* **180**, 89–94.

Holme, I.B., Brinch-Pedersen, H., Lange, M. and Holm, P.B. (2008) Transformation of different barley (*Hordeum vulgare* L.) cultivars by *Agrobacterium tumefaciens* infection of in vitro cultured ovules. *Plant Cell Rep.* **27**, 1833–1840.

Holme, I.B., Dionisio, G., Brinch-Pedersen, H., Wendt, T., Madsen, C.K., Vincze, E. and Holm, P.B. (2012a) Cisgenic barley with improved phytase activity. *Plant Biotechnol. J.* **10**, 237–247.

Holme, I.B., Brinch-Pedersen, H., Lange, M. and Holm, P.B. (2012b) Transformation of barley (*Hordeum vulgare* L.) by *Agrobacterium tumefaciens* infection of in vitro cultured ovules. In *Transgenic Plants: methods and Protocols* (Dunwell, J.M. and Wetten, A.C., eds), pp. 151–161. Totowa, NJ: Humana Press.

Holme, I.B., Dionisio, G., Madsen, C.K. and Brinch-Pedersen, H. (2017) Barley HvPAPhy_a as transgene provides high and stable phytase activities in mature barley straw and in grains. *Plant Biotechnol. J.* **15**, 415–422.

Krishnan, H.B., Kim, W.S., Jang, S. and Kerley, M.S. (2009) All three subunits of soybean β-conglycinin are potential food allergens. *J. Agric. Food Chem.* **57**, 938–943.

Krogh Madsen, C., Pettersson, D., Hjortshøj, R., Katholm, A. and Brinch-Pedersen, H. (2018) Superior growth rates in broilers fed wheat with low in vitro feed-xylanase inhibition. *J. Agric. Food Chem.* **66**, 4044–4050.

Mann, D.G.J., Lafayette, P.R., Abercrombie, L.L., King, Z.R., Mazarei, M., Halter, M.C., Poovaiah, C.R. *et al*. (2012) Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species. *Plant Biotechnol. J.* **10**, 226–236.

Nielsen, P.K., Bønsager, B.C., Fukuda, K. and Svensson, B. (2004) Barley α-amylase/subtilisin inhibitor: structure, biophysics and protein engineering. *Biochim. Biophys. Acta*, **1696**, 157–164.

Nørgaard, J.V., Malla, N., Dionisio, G., Madsen, C.K., Pettersson, D., Lærke, H.N., Hjortshøj, R.L. *et al*. (2019) Exogenous xylanase or protease for pigs fed barley cultivars with high or low enzyme inhibitors. *Anim. Feed Sci. Technol.* **248**, 59–66.

Panting, M., Holme, I.B., Björnsson, J.M., Zhong, Y. and Brinch-Pedersen, H. (2021) CRISPR/Cas9 and transgene verification of gene involvement in unfolded protein response and recombinant protein production in barley grain. *Front. Plant Sci.* **12**, 755788.

Pekkarinen, A.I. and Jones, B.L. (2002) Trypsin-like proteinase produced by *Fusarium culmorum* grown on grain proteins. *J. Agric. Food Chem.* **50**, 3849–3855.

Pekkarinen, A.I., Sarlin, T.H., Laitila, A.T., Haikara, A.I. and Jones, B.L. (2003) Fusarium species synthesize alkaline proteinases in infested barley. *J. Cereal Sci.* **37**, 349–356.

Pope, M., Dean Boyd, R., Holzgraefe, D., Monaco, H. and Sifri, M. (2024) Economic value and environmental impact of soybean meal in poultry and swine diets. *J. Appl. Poult. Res.* **33**, 100449.

Rosenkilde, A.L., Dionisio, G., Holm, P.B. and Brinch-Pedersen, H. (2014) Production of barley endoprotease B2 in *Pichia pastoris* and its proteolytic activity against native and recombinant hordeins. *Phytochemistry* **97**, 11–19.

Shewry, P.R., Bunce, N.A.C., Kreis, M. and Forde, B.G. (1985) Polymorphism at the Hor 1 locus of barley (*Hordeum vulgare* L.). *Biochem. Genet.* **23**, 391–404.

Singh, A., Meena, M., Kumar, D., Dubey, A.K. and Hassan, M.I. (2015) Structural and functional analysis of various globulin proteins from soy seed. *Crit. Rev. Food Sci. Nutr.* **55**(11), 1491–1502.

Soyars, C.L., Peterson, B.A., Burr, C.A. and Nimchuk, Z.L. (2018) Cutting edge genetics: CRISPR/Cas9 editing of plant genomes. *Plant Cell Physiol.* **59**, 1608–1620.

Stein, H.H., Berger, L.L., Drackley, J.K., Fahey, G.C., Hernot, D.C. and Parsons, C.M. (2008) 18 – Nutritional properties and feeding values of soybeans and their coproducts. In *Soybeans* (Johnson, L.A., White, P.J. and Galloway, R., eds), pp. 613–660. Urbana, IL: AOCS Press.

Tanner, G.J., Colgrave, M.L., Blundell, M.J., Howitt, C.A. and Bacic, A. (2019) Hordein accumulation in developing barley grains. *Front. Plant Sci.* **10**, 649.

The European Commission. (2020) *The European green deal*. https://commission.europa.eu/strategy-and-policy/priorities-2019-2024/european-green-deal_en

Wang, T., Qin, G.X., Sun, Z.W. and Zhao, Y. (2014) Advances of research on glycinin and β-conglycinin: a review of two major soybean allergenic proteins. *Crit. Rev. Food Sci. Nutr.* **54**(7), 850–862.

Wang, L., Liu, Y., Zhang, D., Ding, H., Feng, S., Zhao, C., Wu, J. *et al*. (2023) Soybean antigen protein-induced intestinal barrier damage by trigging endoplasmic reticulum stress and disordering gut microbiota in weaned piglets. *Molecules*, **28**(18), 6500.

Zhang, H.Y., Yi, J.Q., Piao, X.S., Li, P.F., Zeng, Z.K., Wang, D., Liu, L. *et al*. (2013) The metabolizable energy value, standardized ileal digestibility of amino acids in soybean meal, soy protein concentrate and fermented soybean meal, and the application of these products in early-weaned piglets. *Asian Australas. J. Anim. Sci.* **26**, 691–699.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Translation of Golden Promise mutant lines.
Figure S2 CI-1A and CI-1B sequence alignments, protospacer comparison and Off-target sequences.
Figure S3 CI-1A Stairway T_0 and T_1 mutation sequences.
Figure S4 Agronomic traits of Stairway ci-1a mutant and Golden Promise ci-1a/1b/2 triple mutant.
Table S1 Immature embryo transformation efficiency of Golden Promise.
Table S2 Regenerated plants from triple mutant transformation and their zygosity.
Table S3 sgRNA oligo sequences used for ligation into entry vector pJG85.
Table S4 Primer sequences and PCR product sizes for target region of all six protease inhibitor genes and hygromycin primers for T-DNA integration screening.