Establishment of regeneration and transformation protocols to create hypoallergenic peanut (*Arachis hypogaea*) and mustard (*Brassica juncea*) through genome editing

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Abstract

A stable and reproducible in vitro regeneration system is necessary for genetic engineering, however, explant responses vary widely between and within species and are highly dependent on the culture conditions. Despite decades of research, plant regeneration is still challenging especially with some plant species referred to as recalcitrant. In this study, we investigated the regeneration and genetic transformation capacities of different lines of peanut (*Arachis hypogaea*) that is considered recalcitrant and Brown mustard (*Brassica juncea*) in order to enable editing of the allergens Ara h 1 and Bra J I in peanut and mustard, respectively, via CRISPR/Cas9. Moreover, we demonstrated that, several of the first edited mustard lines displayed reduced or total absence of the Bra J I protein.

Firstly, the factors affecting the adventitious shoot regeneration of both plant species were investigated using four peanut lines and six mustard lines from two geographical regions (Europe and India). In both species, shoot regeneration was significantly influenced by the explant type and the genetic make-up of the different lines tested. In mustard, the 5 days old cotyledon explants of all lines showed better responses than hypocotyls regarding adventitious shoot regeneration whereas in peanut the leaflet explants of 5-day-old seedlings exhibited superiority. The combinations of different types of cytokinins and auxins were tested on the explants of both plant species.

In all peanut lines, the leaflet explants responded best on medium with 22.19 µM 6-benzylaminopurine (BAP) (+ 2.3 µM kinetin), which was reduced to 7.40 µM BAP after eight weeks, with regeneration rates of 10-89.1% and a mean shoot number per regenerating explant of 1-3.1 shoots. For mustard, the medium containing 8.88 µM BAP, 5.37 µM 1-naphthaleneacetic acid (NAA), and 9.95 µM Silver nitrate ($AgNO_3$) resulted in the highest shoot regeneration rates (58-72% and 65-90% for the European and Indian lines, respectively) as well as the highest shoot numbers per regenerating explant (2.2-2.7 and 2.3-3.0). Furthermore, the effect of different light qualities on shoot regeneration from leaflet explants of the peanut lines was investigated in order to promote shoot induction and elongation. A strong effect of the culture temperature on the regeneration efficiency was observed as different light treatments were connected with different culture temperatures. However, red and blue LEDs could substitute tubular fluorescent lamps without affecting shoot regeneration.

In the second step, *Agrobacterium*-mediated transformation of both plant species was investigated. Transgenic plants carrying large deletions of 566 up to 790 bp as well as indels in the targeted regions especially, indels in all four *Bra j I* alleles were obtained with the mustard lines tested. Seed viability was investigated in several transgenic mustard lines through in vitro and ex vitro germination. A decrease in seed viability and seed formation was observed in some edited lines, which indicated that the mutation of the major allergen Bra J I in mustard affected seed development. Part of the seeds exhibited aberrant phenotypes that resulted in the rupture of the testa already in the siliques. In contrast, the regenerated shoots from the different transformation experiments with various peanut lines exhibited a lack of transgenicity.

Key words: *Agrobacterium tumefaciens*, *Arachis hypogaea*, *Brassica juncea*, CRISPR/Cas, organogenesis.

Zusammenfassung

Ein stabiles und reproduzierbares In-vitro-Regenerationssystem ist für die genetische Transformation von Pflanzen erforderlich, aber die Reaktionen der Explantate variieren stark zwischen und innerhalb von Pflanzenarten und hängen stark von den Kulturbedingungen ab. Trotz jahrzehntelanger Forschung ist die Pflanzenregeneration immer noch eine Herausforderung, insbesondere bei einigen Pflanzenarten, die als schwierig zu regenerieren gelten. In dieser Arbeit wurden Fähigkeit zur Regeneration und genetischen Transformation von verschiedenen Linien von Erdnuss (*Arachis hypogaea*), die als schwierig zu regenerieren eingestuft wird, und Braunem Senf (*Brassica juncea*) untersucht, um die Allergene Ara h 1 und Bra J I in beiden Pflanzenarten jeweils über CRISPR/Cas9 zu editieren. Darüber hinaus wurde gezeigt, dass mehrere der ersten editierten Senflinien eine reduzierte oder völlig fehlende Abundanz des Bra J I-Proteins aufwiesen.

Zunächst wurden Faktoren, die die Adventivsprossbildung beider Pflanzenarten beeinflussen, an von vier Erdnusslinien und sechs Senflinien aus zwei geografischen Regionen (Europa und Indien) untersucht. Bei beiden Pflanzenarten wurde die Sprossregeneration signifikant durch die Explantatarten und die Genotypen der verschiedenen getesteten Linien beeinflusst. Bei Senf zeigten die fünf Tage alte Kotyledonenexplantate aller Linien bessere Reaktionen im Hinblick auf die Adventivsprossbildung als Hypokotylexplantate, während bei Erdnuss Primärblattexplantate von fünf Tage alten Sämlinge gut geeignet waren. Kombinationen verschiedener Arten von Cytokininen und Auxinen wurden an den Explantaten beider Pflanzenarten getestet.

Bei allen Erdnusslinien reagierten die Explantate am besten auf einem Medium mit 22,19 µM 6-Benzylaminopurine (BAP) (+ 2,3 µM Kinetin), dessen Konzentration nach acht Wochen auf 7,40 µM BAP reduziert wurde, mit der höchsten Regenerationsrate von 10-89,1 % und einer mittlere Sprosszahl pro regenerierendem Explantat von 1-3,1 Sprossen. Für Senf zeigte das Medium mit 8,88 µM BAP; 5,37 µM 1-Naphthaleneacetic Acid (NAA) und 9,95 µM Silbernitrat ($AgNO_3$) die höchsten Sprossregenerationsraten (58-72 % und 65-90 % bzw. für die europäische und die indischen Linien) sowie die höchsten Sprosszahlen (2,2-2,7 und 2,3-3,0). Weiterhin wurde der Effekt unterschiedlicher Lichtqualitäten auf die Sprossregeneration an Explantaten der Erdnusslinien untersucht, um die Sprossinduktion und -elongation zu fördern. Ein starker Einfluss der Kulturtemperatur auf die Regenerationseffizienz wurde beobachtet, da unterschiedliche Lichtbehandlungen mit unterschiedlichen Kulturtemperaturen verbunden waren. Rote und blaue LEDs könnten jedoch Leuchtstoffröhren ersetzen, ohne die Sprossregeneration zu beeinträchtigen.

Im zweiten Schritt wurde die *Agrobacterium*-vermittelte Transformation beider Pflanzenarten untersucht. Transgene Pflanzen, die große Deletionen von 566 bis zu 790 bp trugen, sowie Indels in den Zielregionen, insbesondere Indels in allen vier *Bra j I*-Allelen, wurden für die getesteten Senflinien erhalten. Die Keimfähigkeit der Samen wurde in mehreren transgenen Senflinien anhand der In-vitro- und Ex-vitro-Keimung untersucht. Bei einigen editierten Linien wurde eine Abnahme der Keimfähigkeit und des Samenansatzes beobachtet, was darauf hindeutet, dass die Mutation des Hauptallergens Bra J I in Senf die Samenentwicklung beeinträchtigte. Ein Teil der Samen wies abweichende Phänotypen auf, die bereits in den Schoten zum Durchbruch der Testa führten. Im Gegensatz dazu waren die regenerierten Sprosse aus zahlreichen Transformationsversuchen mit verschiedenen nicht transgen.

Schlüsselworte: *Agrobacterium tumefaciens*, *Arachis hypogaea*, *Brassica juncea*, CRISPR/Cas, Organogenese.

Contents

List of abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
$AgNO_3$	silver nitrate
BAP	6-benzylaminopurin
CRISPR	clustered Regularly Interspaced Short Palindromic Repeats
Cas9	CRISPR associated protein 9
DAP	day after pollination
DNA	deoxyribonucleic acid
GFP	green fluorescent protein
IBA	indole-3-butyric acid
HPT	hygromycin phosphotransferase
IMS-PCB	insulated metal substrate printed circuit boards
kDa	kilodaltons
Kin	kinetin
LED	light-emitting diode
MT	meta-topolin
MS	Murashige and Skoog (1962) mineral elements
NAA	1-Naphthaleneacetic acid
NaOCl	sodium hypochlorite
PCR	polymerase chain reaction
PPFD	photosynthetic photon flux density
PAR	photosynthetic active radiation
RNA	ribonucleic acid
SgRNA	single guide RNA
SpCas9	streptococcus pyogenes
Spec	spectinomycin pyogenes Cas9
TALEN	transcription activator-like effector nucleases
T-DNA	transfer DNA
TDZ	thidiazuron
SIM	shoot induction media
TFL	tubular fluorescent lamps
Zea	zeatin

1 General introduction

This thesis focused on the organogenesis and genetic transformation of two plant species with high allergenic potential namely peanut (*Arachis hypogaea*) and Brown mustard (*Brassica juncea*). The gene editing technique CRISPR/Cas9 was used to target the allergens Ara h1 and Bra j I, which are seed storage proteins from peanut and mustard, respectively.

1.1 Production and economic importance of peanut and mustard

1.1.1 Production and economic importance of peanut

Peanut or groundnut (*Arachis hypogaea* L. (Resslar, 1980)) is an essential crop and is cultivated in more than 100 countries under different agroecological environments (Variath and Janila, 2017). Due to polyploidy and sterility barriers, conventional peanut breeding is quite challenging (Wilson et al. 2019). In the year 2021, the global production area was 29.930.000 ha (USDA, www.fas.usda.gov). Nearly 95% of the worldwide production is in developing countries with Asia counting for about 65%. Based on the peanut global output, the major producers are China (36%), India (14%), Nigeria (8%), USA (6%) and Sudan (5%). Over the past 30 years, peanut global production area has increased by about 50% whereas peanut production has more than doubled (increasing by 119%) and reached 50,353,000 metric tons in 2021. The production increment is mainly due to the intensification and mechanization of the production systems, the use of improved varieties, modern crop management practices, and improvement of the irrigation system (Variath and Janila, 2017). However, the productivity levels in that period of time in most of the developing countries have not improved and this is attributed to numerous factors such as, the biotic and abiotic stresses, diverse edaphic characteristics and lack of adequate agricultural policy (Pratap et al., 2018). The complete peanut genome sequence is now available (Bertioli et al., 2019), which is a significant step in creating new varieties with specific traits like hypoallergenic, pest resistance, high nutritional value, high yield and tolerance to abiotic and biotic stressors. Meanwhile, the high-input production system practiced in the USA, China, Brazil, Argentine and Egypt has significantly improved peanut yields from 2 to 4 t/ha. Especially in China, the production area was increased by almost 2-fold, which represented by far the largest peanut producer in 2021 (USDA).

In the international market, peanut is commercially used for the production of oil, flour, butter, snack products and especially for confectionery types. The global peanut market reached a revenue of 3.8 billion dollars in 2021 and is expected to reach 5.3 billion dollars by 2027 (IMARC, https://www.imarcgroup.com) which accounted for nearly 25% of the total edible nut's revenue worldwide (Statista.com). In 2020, Argentina, India, USA, Brazil and Sudan produced about 71% share of the global exports whereas Europe, China, Canada and Australia accounted for about 62% share of the global import in international trade of peanut (www.worldstopexports.com).

1.1.2 Production and economic importance of mustard

Brassica juncea (L.) Czern, generally known as brown, Indian or oriental mustard, is an important edible oilseed crop worldwide (Rancé, 2003). It is widely cultivated, from Africa (northern and western regions) to central Asia (the southern and southeastern regions of the former Soviet Union), to Europe and North America in cool temperate regions and even at high altitudes in the subtropics (Dixon, 2007). During 2010 to 2020, the global production area of mustard has decreased by about 22% to 619,495 ha, whereas the global mustard production has decreased by 13% to 540,454 metric tons (FAO, www.fao.org). In the year 2016–17, Asia (38.31%), the European Union (6.96%), and North America (23.94%) accounted for nearly 70% of the world's production, with Nepal accounting for 67.58% of production in Asia, Canada for 82.01% in North America, and France for 37.72% in European Union. *B. juncea* is mainly grown in Canada and France for condiment purposes and in Australia, New Zealand and India as oilseed crop (Prakash and Hinata 1980; Woods et al., 1991). The overall production of *B. juncea* is mainly affected by biotic and abiotic stresses, such as pathogen attacks, drought, high temperature, frost and salinity (Rai et al., 2022). Moreover, polyploidy, which is defined by interspecific or intergeneric hybridizations followed by chromosomal doubling and could produce significant genetic, genomic, and phenotypic novelty (Soltis et al., 2016, Kyriakidou et al., 2018).

In the international market, mustard seed demand is increasing which is probably due to its use in the food and beverage industry, cosmetic industry, pharmaceutical industry and other industries. The global mustard market reached a revenue of 1.81 billion dollars in 2021 and is expected to reach 2.38 billion by 2027 (www.marketdataforecast.com), which accounts for more than 4% of the global seeds market revenue worldwide (www.statista.com).

1.2 Allergenic potential of peanut and mustard

1.2.1 Allergenic potential of peanut

Peanut allergy is considered as one of the most severe food allergies, affecting nearly 0.6% of the population worldwide (Chen et al., 2018; Palladino and Breiteneder, 2018). Symptoms are triggered after ingestion of the seeds containing the allergenic proteins and can be manifested as severe anaphylaxis (Yocum and Khan, 1994; Palladino and Breiteneder, 2018). Allergenic reactions against peanut are categorized into three groups according to the clinical symptoms, the mild reaction (abdominal pain, irritability, pruritus, urticaria and vomiting), the moderate reaction (breathing difficulty, facial oedema) and severe (collapse/faint, cyanosis and wheeze) (Clarke et al. 1998). Peanut-induced allergy is not a major concern in the Asian and African countries, whereas in the western world and especially in the USA it is considered as a major public health concern, affecting 0.8% of children and 0.6% of adults (Sampson, 2004; Bunyavanich et al., 2014; Du Toit et al., 2015; Nigam, 2015). The processing methods play a determinant role in the allergenicity of peanut allergens. In the western countries, roasted peanuts are mainly consumed while in Asian and African countries, peanuts are mainly boiled and fried. Comstock et al., (2016), reported that standard roasting of peanut actually increases the allergenicity of peanut allergens while frying and boiling decrease the allergenicity. Twelve peanut allergens and multiple isoforms were documented by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Society and are categorized into different food allergen families namely, the Cupin superfamily (Ara h 1, 3), the Prolamin superfamily (Ara h 2, 6, 7, 9), the Profilin family (Ara h 5), the Bet v-1-related proteins (Ara h 8), Oleasin (Ara h 10, 11) and Defensin (Ara h 12, 13) (Mueller et al., 2014). Among the different peanut allergens, Ara h 1, 2, 3 and 6 are recognized by the antibody IgE

(Immunoglobulin E) in over 50% of the peanut-allergic patient and are therefore considered as the major peanut allergens (Koppelman et al., 2001, Mueller et al., 2014). Additionally, according to Lin et al. (2012), peanut-allergic people had noticeably stronger IgE binding to peanut epitopes than peanut-tolerant people, indicating a greater epitope diversity. The study used TileMap to identify 31 IgE-binding areas with numerous epitopes including Ara h 1 (14 regions), Ara h 2 (4 regions), and Ara h 3 (13 regions). Three of the ten linear IgE-binding epitopes that have been identified are immunodominant and are found in exposed, structurally flexible areas of folded proteins (King et al., 2005). Recent research has identified these three IgE-binding epitopes as peptide biomarkers for the diagnosis of clinical peanut allergy and it has been discovered that they are mainly responsible for the cross-reactivity between Ara h 1 and Ara h 2, Ara h 3, and Ara h 6 (Bublin and Breiteneder, 2014). Clinical studied conducted on allergic patients reported that, the allergens Ara h 1, Ara h 2 and Ara h 6 are recognized by about 70-90% of the peanut allergic-patients (Burks et al., 1995; Clarke et al., 1998, Mueller et al., 2014). Most of the major peanut allergens are seed storage proteins (Pedrosa et al., 2012), resistant to digestion (Koppelman et al., 2010) with a molecular mass between 7 to 65 kDa (Boldt, 2005). The ingestion of manufactured food products containing traces of peanut between 100 to 1000 mg by a peanut allergenic individual is sufficient to trigger serious symptoms (Sicherer et al., 2003). Peanut allergy is a lifelong health concern without effective and successful treatment options but with only treatment to mitigate the allergic reactions. Therefore, peanut allergenic individuals must strictly avoid exposure to peanut (Burks et al. 2015). Moreover, using biotechnology-based methods such as RNAi or CRISPR/Cas, might be useful to precisely target allergenic proteins in peanut and could lead to the production of low allergy containing peanut.

1.2.2 Allergenic potential of mustard

The international food allergen labelling regulations, especially in the European Union or Canada declared mustard as one of the priority food allergens (FARRP, https://farrp.unl.edu). The European Union with the Regulation N° 1169/2011 considered mustard as one of the 14 major allergens and therefore it should be clearly indicated in the list of ingredients. This is the

result of serious health issues that mustard allergy represents in many countries, as reported cases of allergic reaction are typically from the consumption of mustard sauce or other products containing mustard in extracts namely ketchup sauces, mayonnaise, dips (Monsalve et al. 2001).

Mustard allergy is known as one of the most frequent spice allergies with severe systematic reactions, namely acute severe urticaria and angioneurotic oedema, facial and throat sweeling, chest tightness, gastric pain, rhinitis, bronchial asthma and anaphylactic shock (Panconesi et al. 1980; Menéndez-Arias et al., 1988 and 1990; Niinimäi et al., 1989; Dominguez et al., 1990; Vidal et al., 1991; Jorro et al., 1995). According to the estimated prevalence of the allergy in France, mustard allergy represents nearly 1% of food allergies in children and almost 7% of the total food allergies (Lietzow, 2021). The consumption of an equivalent of 125 mg of mustard is enough to elicit an allergenic reaction, with 40 mg of mustard seasoning containing approximately 0.8 mg of protein (Morisset et al. 2003; Figueroa et al. 2005).

The allergen Bra j I, a 2S albumin seed storage protein belonging to the napin family is recognized by the IgE of sensitive individuals with a relatively strong response to allergy test. This protein with a molecular weight of nearly 16 kDa is considered as the major allergen in *B. juncea* and is made of two chains (37 and 92 amino acids) linked by disulphide bridges (Gonzalez de la Peña et al., 1991, L'Hocine et al., 2019). Furthermore, five iso-allergenic fractions (*Bra j* IA, IB, IC, ID and IE) of the allergen Bra j I were identified with *Bra j* IE reported to be the most prevalent. These fractions share an epitope containing the sole Tyr residue of the allergen and assumed to be the source of the allergy (Monsalve et al., 1993). Mustard allergenic proteins strongly resist to heat denaturation and are poorly digestible, therefore preventing the reduction of the allergenic potential during mustard processing (Lietzow, 2021). Till date, there is no effective preventive treatments for mustard allergy. Therefore, a mustard sensitive individual must strictly avoid exposure to mustard (Lietzow, 2021).

1.3 The joint project LACoP

This thesis was part of the project LACoP standing for “Low allergen Containing Plants”, a research project initiated by Leibniz University of Hanover and funded by the Federal Ministry of Education and Research (BMBF). The aim of the LACoP project was to reduce or eliminate the allergenic epitopes in plant foods using CRISPR/Cas-based genome editing technology. It was a three-year project, that started in September 2017 and ended in January 2021. Seven project partners (including project coordination) (from all over Germany) were involved. The LACoP project was structured into seven work packages (WPs): (WP1-1) Heterologous expression of the allergens and their deletion variants; test development, (WP1-2) Targeted removal of allergenic components in *Brassica juncea* and *Arachis hypogaea* via genome editing, (WP2) Development of regeneration and transformation protocols for *Brassica juncea* and *Arachis hypogaea*, (WP3) Recombinant antibodies, (WP4) Confirmation of the hypo-allergenicity of the modified peanut and mustard seeds using IgE testing with patient sera, (WP5) Development of an immunoassay for the in vitro diagnosis of allergen-specific IgE antibodies in blood samples and (WP6) Consumer communication - information and feedback, which were all linked to each other.

This thesis was embedded in the project P2 which aimed to establish in vitro regeneration and genetic transformation protocols for *Brassica juncea* and *Arachis hypogaea*. Therefore, experiments to optimize the shoot regeneration of mustard were conducted (Assou et al., accepted, chapter 2), the results of which were used for the genetic transformation of mustard (Assou et al., 2021, chapter 3). Moreover, experiments on cytokinins and light quality on adventitious shoot regeneration of peanut were as well conducted (Assou et al., 2022, chapter 4), which outcomes were applied to intense experiments aiming at genetic transformation of peanut. The establishment of in vitro regeneration and genetic transformation protocols of both plant species is necessary to remove the allergy inducing areas of the Ara h 1 and Bra J I using genome editing technology like CRISPR/Cas9.

1.4 In vitro regeneration and genetic transformation of peanut and mustard

A successful In vitro regeneration system is based on the totipotency of the plant cells under specific culture conditions (Skrzypek et al., 2012). Several factors such as the genotype, the explant type, the age of explants, the culture condition, the plant growth regulators (PGRs) and the culture media greatly influence the success of in vitro plant regeneration processes (Pierik, 1997). For model breeding techniques like CRISPR/Cas, it is essential to establish a stable and reproducible shoot regeneration system through the optimization of these different factors.

1.4.1 In vitro regeneration and genetic transformation of peanut

Extensive investigations were conducted in the recent years, leading to successful establishment of in vitro regeneration of peanut through organogenesis (Table 1) from numerous explants such as cotyledon, cotyledonary nodes, petioles, leaflets, epicotyl and hypocotyl. However most of the studies reported variable regeneration efficiency ranging between 7 to 80%.

In most studies, the in vitro regeneration system of peanut is characterized by, the use of Murashige and Skoog (1962) medium, generally supplemented with B5 vitamins (Gamborg et al., 1968) Auxin and cytokinin, which are essential to induce morphogenesis with the cytokinins benzylaminopurine (BAP) and thidiazuron (TDZ) often used in high concentrations ranging from 6.66 µM to 110 µM (Cheng et al., 1992; Kanyand et al., 1994; Pestana et al., 1999; Akasaka et al., 2000; Tiwari and Tuli 2012; Tiwari et al., 2015) whereas the most commonly used auxins were 2,4-D (2,4-dichlorophenoxy acetic acid) and NAA (naphthalene acetic acid) (Cheng et al., 1992; Tiwari et al., 2015). The commonly used light source were fluorescent tubes (FLs) with a photon flux density ranging of 12-130 µmol m^{-2} s^{-1}, a photoperiod of 16h and a temperature of 24-35°C.

Table 1. Overview of the literature on shoot organogenesis in several *Arachis hypogaea* genotypes, using different explant types.

Variety	Explants	Age	Plant growth regulators	% of regeneration	Reference
Cv. Colorado Manfredi	Leaflets	3-5 days old	NAA (5.7 µM) and BA (3.96 µM)	28.3 %	Mroginski et al. (1981)
Virginia type `Chiba-Handachi' Spanish type `Java n°13'	Cotyledonary node	7 days	BA (285 µM)	40-80 %	Daimon and Mii, (1991)
New Mexican Valencia A	Petiolule	8-11 days old	NAA (5.7 µM) and BA (39.6 µM)	52%	Cheng et al. (1992)
Valencia Cv. 'New Mexico'	Leaf, petiole, hypocotyl, Cotyledon	8 days old	TDZ (45.4 µM)	20-70 %	Kanyand et al. (1994)
Valencia Cv. Tatu Spanish	Leaflets, cotyledon	2 or 10 days old	BA (22 µM) and $AgNO_3$ (5µM)	40-56 %	Pestana et al. (1999)
Cv. `Chico`	Leaflets	6-8 days old	TDZ (45.4 µM)	13.2 %	Akasaka et al. (2000)
Spanish 'JL-24'	Cotyledon	1 day old	2,4-D (10 µM) and BA (20 µM)	80 %	Sharma and Anjaiah, (2000)
JL-24; TMV-2; TAG-24; Dh-3-30	Leaflets	1 day old	NAA (4.95 µM) and BA (13.32 µM)	77-81 %	Tiwari and Tuli, (2009)
Virginia-type Cv. Luhua no. 14	Epicotyl	8 days old	NAA (4.30 µM) and BA (44.39 µM)	80.7 %	Shan et al. (2009)
Var. GG20	Leaflets	7 days old	NAA (2.68 µM) and BA (13.32 µM)	70 %	Vadawale et al. (2011)
Valencia Cv. 'New Mexico'	Cotyledon	1 day old	2,4-D (13.57 µM) and BA (19.8 µM)	60 %	Matand et al. (2013)
Runner type 'Florida-07' and 'Georgia Green'	Cotyledon	1 day old	2,4-D (10 µM) and BA (20 µM)	7-24 %	Burns et al. (2012)
New Mexican Valencia A	Cotyledonary node	3 weeks old	BA (6.6 µM)	90 %	Hsieh et al. (2017)

Molecular breeding techniques might be essential in creating new genetic diversity in peanut, and were considered to be very useful in developing cultivars with improved traits such as drought tolerance, resistance to rust, virus, insect and fungus, yield and grain quality (Ahmar

et al., 2020). In peanut, the biolistics/bombardment technique was used to achieve the first successful transformation (Ozias-Akins et al., 1993). Afterwards, several transformation protocols were developed based on biolistic methods targeting embryonic tissues (Chu et al., 2013). In the study of Brar et al. (1994), a stable integration of the herbicide resistance *bar* gene into the embryonic axes of the peanut cultivars 'Florunner' and 'Florigiant' using particle bombardment with a transformation efficiency of 44% was reported. The same method was also used by Yang et al. (1998) and Higgins et al. (2004) to produce resistant transgenic peanut to hygromycin and, respectively to the viruses TSWV (Tomato Spotted Wilt Virus) using immature cotyledons of the peanut cultivars 'Florunner' and 'Georgia Runner' and PStV (Peanut Stripe Virus) using immature leaflets of the peanut cultivars 'Gajah' and 'NC7' with a transformation frequency of 60 to 63%. Moreover, Chu et al. (2008) used biolistic method to silence the allergen Ara h 1 in peanut cultivar 'Georgia Green' through the integration of the plasmid pPPCH-V-Arah1_si carrying hygromycin resistance gene and RNAi construct in immature leaflet.

On the other hand, reports of *Agrobacterium tumefaciens*-mediated transformation have also been made, with success rates that are highly dependent on the genotype, culture conditions, cocultivation and selection protocols, and host-pathogen interactions (Holbrook et al. 2011). Li et al. (1997), reported the expression of the sense RNA of key viral genes of TSWV in transgenic peanut plants of cultivar 'New Mexico Valencia A', using the *Agrobacterium* strain EHA 105 harboring a plasmid with kanamycin resistance gene on immature leaflets with a transformation efficiency of 1.5% whereas Bag et al. (2007) produced transgenic peanut plants of cultivar 'JL-24' with sense and antisense coat protein genes of *Tabacco streak virus* (TSV) using the *Agrobacterium* strain LBA4404 carrying a plasmid with kanamycin resistance gene on de-embryonated cotyledons with a transformation frequency of 15%. Furthermore, *Agrobacterium*-mediated transformation of de-embryonated cotyledons and hypocotyl explants were described respectively by Tiwari et al. (2008) resulting in the production of the first transgenic peanut plants of cultivar 'JL-24' harboring the synthetic *cry1EC* gene for resistance against *Spodoptera litura* and the hygromycin resistance gene using the

Agrobacterium strain EHA 101 with a transformation efficiency of 4%. Moreover, transgenic peanut plants of variety 'Georgia green' with low Ara h 2 content and resistance to kanamycin were produced using the *Agrobacterium* strain EHA 105 with a transformation frequency of 44% (Dodo et al. 2008).

1.4.2 In vitro regeneration and genetic transformation of mustard

As one of the most important *Brassica* species, several researchers worldwide have broadly investigated the in vitro regeneration potential of *B. juncea*. Numerous reports described the shoot organogenesis in several *B. juncea* genotypes, using different explant types mostly prepared from seedlings (Table 2). Nevertheless, due to the factors genotype and explant type, significant variation in shoot regeneration efficiencies (ranging from 0 to 94%) was observed in most of these studies.

The commonly used media for the shoot regeneration system of *B. juncea* were based on the basic Murashige and Skoog (1962) medium, supplemented with B5 vitamins (Gamborg et al., 1968) with different types of auxins (2,4-D (2,4-dichlorophenoxy acetic acid) or NAA (naphthalene acetic acid)) and cytokinins (benzylaminopurine (BAP), thidiazuron (TDZ) and Kinetin (Kn)). In order to improve the shoot morphogenesis in *B. juncea*, the ethylene receptor blocker silver nitrate ($AgNO_3$) was as well used in most studied. The commonly used light source were fluorescent tubes (FLs) with a photon flux density ranging from 50-200 µmol m^{-2} s^{-1}, a photoperiod of 16h and a temperature of 24-28°C.

Table 2. Overview of the literature on shoot organogenesis in several *B. juncea* genotypes, using different explant types.

Variety	Explant	Age	Plant growth regulators	% of regeneration	Reference
Var. Rai-5	Cotyledon	8 days old	NAA (5.7 µM) and BA (3.96 µM)	66 %	George et al. (1980)
CPI 96852 (China) CPI 81793 (India)	Cotyledon	8 days old	NAA (5.7 µM) and BA (3.96 µM)	12 % (China) 55 % (India)	Fazekas et al. (1986)
Cv. RH 36	Cotyledon	6 days old	IAA (5.7 µM) and BA (8.8 µM)	66 %	Narasimhulu and Chopra (1988)
Cv. RLK-81-1	Cotyledon	5 days old	BA (5 µM)	80 %	Sharma et al. (1990)
Var. tsatsai	Cotyledon	6 days old	TDZ (4.54 µM) and NAA (5.37 µM)	67.9 %	Guo et al. (2005)
Cv. BARI sarisha-10	Cotyledon	4 days old	NAA (5.7 µM), BA (3.96 µM) and $AgNO_3$ (11.77 µM)	86.67 %	Bhuiyan et al. (2009)
BINA Sarisha - 4	Cotyledon	4 days old	NAA (3.75 µM), BA (3.96 µM) and $AgNO_3$ (11.77 µM)	33.33 %	Biswas et al. (2017)
Indian mustard	Cotyledon	4 days old	NAA (0.57 µM), BA (3.96 µM)	63 %	Kashyap et al. (2019)
Var. NRCDR-2	Cotyledonary petiole	5 days old	NAA (0.57 µM), BA (3.96 µM) and $AgNO_3$ (58.8 µM)	93.34 %	Thakur et al. (2013)
Var. NRCHB-10	Cotyledonary petiole	5 days old	NAA (1.14 µM) and BA (7.92 µM)	56.5 %	Tiwari et al. (2019)
Cv. RLM 198	Hypocotyl	5 days old	NAA (5.7 µM), BA (3.96 µM) and $AgNO_3$ (20 µM)	72.6 %	Pental et al. (1993)
Var. Pusa Jaikisan	Hypocotyl	10 days old	NAA (3.75 µM) and BA (7.92 µM)	33.3%	Trivedi and Dubey, (2014)
RSPR 03 and RSPR 01	Hypocotyl	10 to 20 days old	2,4-D (0.9 µM) and BA (7.92 µM)	60-70%	Lone et al. (2017)
Pusa bold	Peduncle	-	NAA (5.7 µM), BA (3.96 µM) and $AgNO_3$ (30 µM)	81 %	Eapen and George, (1997)

The tremendous progress in molecular biology provides different approaches for plant genetic engineering to develop transgenic *B. juncea* plants with improved breeding traits. In the last decades, several studies dealing with the introduction of various new traits in *B. juncea* were reported. With regard to the improvement of the nutritional aspect of *B. juncea*, Augustine et al. (2013) applied RNAi-based target suppression of *BjMYB28* gene to develop low glucosinolate *B. juncea* lines resulting in the significant improvement of the seed meal quality whereas hairpin-RNA was used by Sinha et al. (2007) to silence the *fatty acid elongase* gene, which reduced the production of erucic acid in *B. juncea*. Moreover, Yusuf and Sarin (2007) through genetic engineering methods successfully overexpressed the *γ -tocopherol methyl transferase* (*γ-TMT*) gene to increase the vitamin E (α-tocopherol) level in the seeds of *B. juncea*. Das et al. (2006) and Kanrar et al. (2006) reported successful modification of the oil composition leading to the production of gamma-linolenic acid in *B. juncea*. Concerning biotic and abiotic stresses, Østergaard et al. (2006) developed transgenic *B. juncea* plants with pod shatter-resistance through the ectopic expression of the *FRUITFULL* gene, whereas salt tolerant and herbicide tolerant transgenic *B. juncea* were developed, respectively by Prasad et al. (2000) and Bisht et al. (2004). Furthermore, the development of insect resistant transgenic *B. juncea* (Dutta et al., 2005; Cao et al., 2008; Rani et al., 2017) and heavy metal tolerant transgenic *B. juncea* (Zhu et al., 1999; Pilon-Smits, 2005; Gasic and Korban, 2007; Bhuiyan et al., 2011) were as well reported.

1.5 Objectives of the thesis

This thesis aimed at identifying the major factors affecting the in vitro regeneration and genetic transformation for peanut and mustard in order to establish and improve protocols. These protocols are essential for the reduction or removal of the allergenic epitopes of the allergens Ara h 1 and Bra J I through genome editing using CRISPR/Cas9. This endeavor was split into two main goals: identifying the different factors involved in the establishment of an efficient In vitro regeneration system via adventitious shoot formation of peanut and mustard (Chapters 2 and 4), which is a prerequisite for developing *Agrobacterium tumefaciens*-based gene transfer systems for mustard (Chapter 3).

First of all, we aimed at selecting in vitro lines of both plant with a high regeneration response. A total of nine *B. juncea* lines (6 European lines and 3 Indian lines) and four *A. hypogaea* lines were investigated. Based on the literature, factors such as the explant type, the age of explants, the plant growth regulators (PGRs) and the culture condition, were examined. Shoot regeneration efficiencies were calculated based on the mean number of explants regenerating adventitious shoots and the mean number of shoots per regenerating explant. The results of these experiments are summarized in chapters 2 and 4.

Then, two responsive out of the nine *B. juncea* lines (1 European line and 1 Indian line) and the four *A. hypogaea* lines were selected for *Agrobacterium tumefaciens*-mediated transformation. Thereby, it was intended to use the gene editing technology CRISPR/Cas9 to remove the major allergens Ara h 1 and Bra J I, respectively, from *A. hypogaea* and *B. juncea*. From these experiments, only transgenic *B. juncea* plants showing partial or total removal of the allergen Bra j I from both selected lines were obtained. These results are described in the publication entitled “Removing the major allergen Bra j I from brown mustard (*Brassica juncea*) by CRISPR/Cas9” (Assou et al., 2021, chapter 3).

The deletion of the allergen Bra J I, the most abundant seed storage protein in mustard, prompted us to investigate the embryonic development of seeds from these transgenic mustard plants (T_1 and T_2 generations) and compared them to the wild-type plants to detect any abnormalities in seed development caused by the mutation (genetic transformation). Therefore, ovules were taken at different time points after pollination and prepared for DIC microscopy to scrutinize any developmental abnormality. The results of this experiment are summarized in the general discussion.

2 Optimization of in vitro adventitious shoot regeneration in *Brassica juncea* L. of different origins for application in genetic transformation and genome editing

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Abstract

Besides being an important oilseed crop, *Brassica juncea* is used as leafy vegetable, spice and biofumigation crop. To use novel breeding techniques such as genome editing, an efficient, reproducible regeneration protocol is beneficial. For *B. juncea*, adventitious shoot regeneration had been described previously, but mainly using lines of the Indian and Chinese gene pool. In this study, we aimed at establishing and improving an adventitious shoot regeneration protocol for current *B. juncea* cultivars and breeding lines from the European gene pool ('Terraplus', 'Terratop', 'Terrafit', 'Energy', SFB18/1 and SFB22/15) and compared their response to gene bank accessions originating from India. Cotyledon and hypocotyl explants of five days-old seedlings were cultured on Murashige and Skoog medium supplemented with different concentrations BAP, IBA and NAA with or without 9.95 µM $AgNO_3$. When the cotyledon and hypocotyl explants were cultured on media supplemented only with BAP and IBA, the regeneration rate was below 50% and 5% respectively. The highest shoot regeneration rates of 58-72% and 65-90% for the European and Indian lines, respectively, as well as the highest shoot number of 2.7 and 3.0 per regenerating explant were obtained on a medium containing 8.88 µM BAP, 5.37 µM NAA and 9.95 µM $AgNO_3$. Shoot regeneration rate was severely affected in all lines by ethylene production resulting from the addition of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid to the medium. Also, $AgNO_3$ caused an increased ethylene production measured after ten days of culture, but it had a significant positive effect on shoot regeneration indicating a negative effect of ethylene on early events in organogenesis.

Keywords: *Brassicaceae*, brown mustard, ethylene, organogenesis, plant growth regulators, silver nitrate ($AgNO_3$).

1 Introduction

The genus *Brassica,* belonging to the angiosperm family Brassicaceae and counting 37 species, contains some of the most important oilseed crops worldwide, among them, *Brassica juncea* L. (Gomez-Campo, 1980, Sharma et al., 2012). *B. juncea* is an annual, self-pollinated plant and an amphidiploid (AABB, 2n=36), derived from the hybridization between two diploid progenitors *Brassica rapa* (AA genome, 2n = 20) and *Brassica nigra* (BB genome, 2n = 16) (Yang et al., 2016). The center of origin is supposed to be central Asia and wild forms of *B. juncea* were reported to occur in the Near East and in southern Iran (Rakow, 2004).

Besides the use as oilseed crop, the four subspecies of *B. juncea* offer a rich diversity in morphology as well as quality characteristics and are used as leafy vegetable in Asia (ssp. *integrifolia*), oilseed and fodder (ssp. *juncea*), root-tuber vegetable (ssp. *napiformis*), and leafy vegetable (ssp. *taisai*) (Spect and Diederichsen, 2001). In North America and Europe, *B. juncea* is cultivated for the seed and for producing mustard as a condiment, while, in the Indian subcontinent it is grown for the seed oil, and in the Far East as a vegetable (Vaughan, 1977). Furthermore, it is a valuable component in biodiesel owing to its high content of erucic acid (Premi et al., 2013). In addition, glucosinolate-rich *B. juncea* highly contribute to biofumigation effects and can be used to mitigate the effects of soilborne pathogens and replant diseases (Mattner et al., 2008; Yim et al., 2016; Hanschen and Winkelmann, 2020).

The seed of *B. juncea* is rich in fatty acids and represents an excellent reservoir for natural antioxidants and vitamin E (Kaur et al., 2019). Nevertheless, its high glucosinolate content and the fatty acid composition represent a health issue for consumption (Saikia et al., 2018; Yashpal et al., 2020). In the Indian *B. juncea* lines, 3-butenyl glucosinolate is the dominant glucosinolate, whereas, the Chinese *B. juncea* seed contains high concentrations of 2-propeny (allyl) glucosinolate (Rakow, 2004). Lines of both origins are also characterized by a high erucic acid content (40 to 45 % for the Indian and 30 to 35 % for the Chinese forms, respectively) (Rakow, 2004). In Canada, breeding addressed these health issues and resulted in improved oilseed *B. juncea* without erucic acid and a fatty acid composition very similar to that of *B.*

napus (canola). In addition, the seed meal has only minor concentration of allyl glucosinolate (<3 µmol g^{-1} seed) and a low total glucosinolate content (Rakow, 2004).

A third health issue if it comes to human consumption, is the allergenic potential of mustard. In order to develop low-allergenic genotypes, Assou et al. (2021) used CRISPR/Cas9 to remove a seed storage protein, Bra J I from *B. juncea.* Although the above-mentioned breeding approaches represent significant progress toward the development of safer food crop, further breeding is needed to develop cultivars with high yields, tolerance to abiotic and biotic stressors and high nutritional value for being utilized as food and feed (Thakur et al., 2020). Therefore, breeding methods that specifically target genes underlying important traits are of importance. Since the genome sequence of *B. juncea* is available (Yang et al., 2016), future breeding will also benefit from the outstanding potential of genome editing which has already been employed in first studies Assou et al. (2021).

To exploit the potential of biotechnology in *B. juncea*, an efficient and reproducible tissue culture system is crucial, which is a prerequisite for the majority of approaches aiming at genetic transformation and gene editing. For some species of the Brassicaceae, such as *Arabidopsis thaliana* (Clough and Bent 1998) and *Brassica napus and B. carinata* (Verma et al. 2008), the floral dip approach was successful and efficient and replaced the tissue culture-based transformation methods. However, despite intense efforts to use floral dip for other plant species, the number of crops amenable to this simple protocol is still very limited.

Adventitious shoots can be induced from different plant tissues and organogenesis is commonly used as regeneration system in *Agrobacterium tumefaciens*-mediated transformation. In *B. juncea*, adventitious shoots were regenerated from different explant types often prepared from seedlings, namely cotyledons (George and Rao, 1980; Fazekas et al., 1986; Narasimhulu and Chopra, 1988; Sharma et al., 1990; Dutta et al., 2005; Guo et al., 2005; Das et al., 2006; Bhuiyan et al., 2009a; Rani et al., 2017; Kashyap and Tharannum, 2019; Naeem et al., 2020; Shyam et al., 2021), cotyledonary petioles (Thakur et al., 2013; Tiwari et al., 2019), hypocotyls (Pental et al., 1993; Pua and Chi, 1993; Guo et al., 2005; Nehnevajova

et al., 2007; Trivedi and Dubey, 2014; Lone et al., 2017; Goswami et al. 2018), leaves (Eapen and George, 1996; Dutta et al., 2008), peduncle segments (Eapen and George, 1997) and transverse thin cell layers (Bhuiyan et al., 2009b). Pronounced genotypic differences for both the percentage of regenerating explants and the number of shoots formed per explant, as well as a considerable variation in the shoot regeneration frequency of the different types of explants were observed in most of these studies. Interestingly, adventitious shoot regeneration of Indian *B. juncea* lines was described to be generally higher than of European/Chinese lines (Fazekas et al., 1986). The Indian lines of *B. juncea* used by Fazekas et al. (1986) had lower oil contents, higher erucic acid and glucosinolate contents and a brown testa, whereas the European and Chinese lines were characterized by a high oil content, a low glucosinolate content and a yellow testa. As introduced before, also the composition of the types of glucosinolates of both groups differ (Rakow, 2004).

The gaseous plant hormone ethylene, which is produced by plant tissues grown in vitro and accumulates in the sealed containers, inhibits cell differentiation and also influences plant growth and development (Biddington, 1992; Pua and Chi, 1993). The ethylene biosynthesis is up-regulated after wounding which is inevitable during explant preparation and results in poor regeneration potential of a variety of plant species (Wang et al., 2002). Blocking the ethylene action, for example by inhibiting the perception by silver nitrate ($AgNO_3$) has been reported to positively affect organogenesis in Indian lines of *B. juncea* (Pua and Chi, 1993; Bhuiyan et al., 2009a).

In this study, we report the establishment of an efficient plant regeneration system in *Brassica juncea* (L.) employing lines of different geographic origins with a focus on the presumably more recalcitrant European lines, through direct organogenesis using cotyledon explants. Therefore, several factors namely the line, plant growth regulators (PGRs), the explant type as well as the use of the ethylene action inhibitor $AgNO_3$ in the regeneration medium were investigated. Finally, the negative effects of ethylene during early stages of adventitious shoot regeneration were demonstrated.

2 Materials and methods

2.1 Plant materials

Seeds of eight brown mustard (*Brassica juncea* L.) lines were used in the experiments. Six European cultivars and breeding lines ('Terraplus', 'Terratop', 'Terrafit', 'Energy', SFB18/1 and SFB22/15), were kindly provided by the breeder P.H. Petersen Saatzucht Lundsgaard GmbH, Grundhof, Germany. Two Indian lines of *Brassica juncea* (L.) Czern. with the accession numbers CR2649 and CR2664 were obtained from the gene bank of the IPK Gatersleben, Germany.

The seeds were surface-disinfected by submerging in 1% NaOCl solution with about 0.1% Tween 20 followed by continuous agitation for 5 min, and washed three times with sterile deionized water under aseptic conditions. The treated seeds were then germinated for five days at 24°C in darkness in 150 mL glasses (ten seeds per vessel), each containing 25 mL germination medium. The germination medium was plant growth regulator-free, consisted of half-strength Murashige and Skoog MS (Murashige and Skoog, 1962) salts and full-strength MS vitamins with 3% (w/v) sucrose, 0.2% (w/v) glucose and was solidified with 0.4% (w/v) gelrite (Duchefa, Haarlem, The Netherlands) (pH adjusted before autoclaving: 5.5-5.6).

2.2 Explant preparation and culture conditions

Explants were gained from the five days old seedlings: the cotyledons were excised and vertically divided into two halves and 5 mm hypocotyl segments were prepared. Care was taken to remove the apical shoot meristems in order to ensure true organogenesis.

The hypocotyl segments were placed horizontally on the surface of the medium, while the cotyledon explants were cultivated with their abaxial side in contact with the medium. Explants were incubated in Petri dishes (90*15 mm) at 24 ± 1°C with a 16 h photoperiod at a photon flux density (PPFD-PAR) of 35-40 µmol m^{-2} s^{-1} provided by two tubular fluorescent lamps (Philips MASTER TL-D 58W/865) for four weeks. Five days after culture induction, cotyledon explants which had lost contact with the medium due to bending were correctly placed on the medium again.

2.3 Culture media for induction of adventitious shoots

For induction of adventitious shoot formation full-strength MS medium with 3% (w/v) sucrose and 0.8% (w/v) Plant agar (Duchefa, Haarlem, The Netherlands) was used, supplemented with different combinations and concentrations of plant growth regulators, namely the cytokinin 6-benzylaminopurine (BAP: 2.22 µM, 4.44 µM, 8.88 µM and 17.76 µM) and the auxins indole-3-butyric acid (IBA: 0.49 µM and 4.92 µM) and naphthalene acetic acid (NAA: 5.37 µM) and the ethylene receptor blocker silver nitrate ($AgNO_3$: 9.95 µM) (Table 1).

Table 1. Plant growth regulator combinations tested for adventitious shoot regeneration from cotyledon and hypocotyl explants of the different lines of *Brassica juncea.* BAP: 6-benzylaminopurine, NAA: 1-naphthalene acetic acid, IBA: indole-3-butyric acid, $AgNO_3$: silver nitrate. SIM: shoot induction medium

	Plant growth regulator concentration (µM)					
Medium	BAP	NAA	IBA	$AgNO_3$	ACC	Used in experiment
SIM 1	2.22		0.49			1
SIM 2	4.44					1
SIM 3	8.88		0.49			1
SIM 4	17.76		4.92			1,2
SIM 5	8.88	5.37		9.95		2,3
SIM 6	8.88		0.49	9.95		2
Control	8.88	5.37				3
ACC	8.88	5.37			500	3

To investigate the effect of ethylene on adventitious shoot formation, five days old cotyledon explants were cultured on the optimum regeneration medium SIM 5 without $AgNO_3$ supplemented with the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid at 500 µM) and the ethylene action inhibitor $AgNO_3$ at 9.95 µM.

For the media preparation, all constituents except $AgNO_3$ and ACC were mixed and adjusted to a pH value of 5.8 before autoclaving for 20 min at 1 bar and 121°C. The $AgNO_3$ and ACC were filter-sterilized (0.22 µm) and added to the medium after autoclaving.

2.4 Experimental set up

Experiment 1: Effects of plant growth regulators and lines

To compare shoot regeneration of the eight brown mustard lines, different combinations of BAP and auxins at different concentrations were tested. In four repetitions of this experiment with three Petri dishes and five explants per Petri dish, a total of 60 hypocotyl and 60 cotyledon explants were cultured on the four media SIM 1, SIM 2, SIM 3 and SIM 4 (Table 1).

Experiment 2: Testing lower BAP concentrations and silver nitrate

To limit the risk of somaclonal variation, in this experiment the concentration of BAP was reduced. Additionally, the promoting effect of the ethylene action inhibitor ($AgNO_3$) on the shoot regeneration that has been documented in literature was tested for the five most responsive brown mustard lines (three European and two Indian). Again, this experiment was carried out four times with three Petri dishes containing the most suitable explant type for shoot induction (five cotyledon explants each). Based on the report of Pua and Chi (1993), three different medium which were developed for Indian mustard were compared (SIM 4, a medium without $AgNO_3$, and two media supplemented with $AgNO_3$, SIM 5 and SIM 6. (Table 1)).

Experiment 3: Effect of ethylene on adventitious regeneration

For this experiment, three media (control: 8.88 µM BAP + 5.37 µM NAA, ACC: 8.88 µM BAP + 5.37 µM NAA + 500 µM ACC and $AgNO_3$ (SIM 5): 8.88 µM BAP + 5.37 µM NAA + 9.95 µM $AgNO_3$) and a total of 59 cotyledon explants obtained from the four most responsive European brown mustard lines were used per variant. In order to measure ethylene concentrations in the headspace, three Petri dishes with eight cotyledon explants each (resulting in 24 cotyledon explants per medium variant and line) were prepared, and ethylene was measured after two and ten days as described below. In addition, for evaluating adventitious shoot regeneration, 35 cotyledon explants per variant (seven Petri dishes with five cotyledon explants each) were

prepared. Thus overall, 59 explants were observed per medium variant and line in this experiment which was performed once.

2.5 Evaluation of adventitious shoot regeneration

All cultures were monitored weekly for contaminations and contaminated Petri dishes or explants were discarded. The numbers of evaluated, non-contaminated explants and Petri dishes are given in Supplemental Tables 1, 2 and 3 for experiments 1, 2 and 3, respectively. After two and four weeks, explants were evaluated for adventitious shoot formation (yes/no) and adventitious shoot formation [%] was calculated per Petri dish (= replicate) by dividing the number of explants with adventitious shoots by the total number of explants in the Petri dish. Additionally, after four weeks, the number of regenerating shoots for every shoot forming explant was recorded. The mean number of adventitious shoots was based on the number of shoots forming explants (=replicate number).

2.6 Ethylene measurement

The Petri dishes, from which headspace samples were taken for the ethylene measurements were prepared as follows: in the lid of each Petri dish, a septum was prepared under aseptic conditions and covered with an adhesive tape on which the exact position of the hole was marked and sealed with silicon lined membrane cap to ensure it was airtight. After two and ten days, ethylene was measured.

All collected headspace samples, and ethylene standards of 10 and 100 ppm were prepared in triplicate as well as ambient air controls. Standard blanks and samples taken directly from the Petri dishes (500 µL gas sample) through the self-prepared septum with a hypodermic syringe were analyzed in less than 24 h. The samples analysis was performed with a gas chromatography (8860 GC system, Agilent, USA) equipped with a Porapak Q (Agilent part no. G3591-81013) packed column and coupled to a flame ionization detector (FID). Helium was used as the carrier gas and the flow rate was maintained constant at 19.8 mL min^{-1}. The injector, the oven and the detector were maintained at a temperature of 100°C, 60°C, and 275°C, respectively. The total analysis time was seven min per sample. The ethylene

quantification was performed at the Institute of Horticultural Production Systems in the department of Fruit Science at Leibniz Universität of Hannover.

2.7 Statistics

Statistical analysis was conducted using the R program (R Core Team, 2021). Raw data was prepared for statistical analysis by using the packages tidyverse (Wickham et al., 2019) and dplyr (Wickham et al., 2021). Numbers of regenerating explants (I) and numbers of shoots per regenerating explant (II) were analyzed as count data in generalized linear models (glm). Deviance analysis was conducted under the assumption of a binomial distribution (I) or a quasi-poisson distribution (II) of data, respectively. Within the glm, the factors line, medium and repetition were specified. For (I) all the factor´s interactions and for (II) only the interaction between factors line and medium were included in the model. Ethylene concentrations were analyzed as logarithmic transformed data (log(x)) within linear models separately for the timepoints of measurement (two and ten days). Factors line and medium and their interaction were set within the models. Quantile-quantile plots were created to check normal distribution of residuals. Analysis of variance (ANOVA) was conducted to check significance of the factors and/or their interaction. For count data as well for metric data pairwise comparisons after Tukey were conducted using the package emmeans (Lenth, 2022). The boxplot for ethylene concentrations was generated using the package ggplot2 (Wickham, 2016).

3 Results

Experiment 1: Optimization of plant growth regulators for adventitious shoot regeneration in different *B. juncea* lines.

The first experiment aimed at identifying the explant type and plant growth regulator combination that are most suited for the adventitious shoot regeneration of the eight brown mustard lines of *B. juncea*. Therefore, different combinations of the plant growth regulators BAP and IBA were compared using hypocotyl and cotyledon explants of five-days old, dark-grown seedlings (Table 1, Fig. S1). Three to four days after culture initiation, the explants had swollen, followed by callus formation after six to seven days, and within ten to fourteen days

the shoot bud initiation started. The adventitious shoot regeneration was markedly influenced by all three factors, the explant type, the line and the plant growth regulator combination.

Considering the explant type, a clear differentiation was possible, because shoot regeneration was observed on cotyledon explants, while hypocotyl explants produced almost no shoots, but callus and roots (Supplemental Fig. S2). In the very rare cases and independent of the medium used, (28 hypocotyl explants formed shoots out of 691 hypocotyl explants in all line x medium combinations), where shoot formation was observed from hypocotyls, these single shoots developed very fast suggesting their origin to be the apical meristem of the seedling. In contrast, the cotyledon explants of all lines, increased in size, started to form callus after one week and subsequently shoot buds. Media containing 4.44 µM or 8.88 µM BAP resulted in root formation (recognized by pronounced root hairs) after 4 weeks (Supplemental Fig. S3). Adventitious shoots were observed about ten to fourteen days after culture initiation. It was important that the proximal side of the cotyledon explants was in good contact with culture medium.

From the four plant growth regulator combinations tested, the combination used in medium SIM 4 (17.76 µM BAP and 4.92 µM IBA) resulted in the highest shoot regeneration frequencies for seven of the eight lines as indicated by the grey cells in Table 2, with maximal values of 47.3% and 46.0%, respectively, for the European line ‘Terratop’ and the Indian line CR2664.

Table 2. Effect of plant growth regulators on adventitious shoot regeneration from cotyledon explants of eight lines of *Brassica juncea* (six European lines: 'Terraplus', 'Terratop', 'Terrafit', 'Energy', SFB18/1 and SFB22/15 and two Indian lines: CR2649 and CR2664) (Experiment 1). MS salts and vitamins were used in all media and explants were cultivated for 4 weeks on the different media. SIM 1: 2.22 µM BAP + 0.49 µM IBA, SIM 2: 4.44 µM BAP + 0.49 µM IBA, SIM 3: 8.88 µM BAP + 0.49 µM IBA, SIM 4: 17.76 µM BAP + 4.92 µM IBA. The replicate numbers are specified in Supplemental Table S1).

	Shoot regeneration (%)				Mean number of shoots per regenerating explant			
Line	SIM 1	SIM 2	SIM 3	SIM 4	SIM 1	SIM 2	SIM 3	SIM 4
'Terraplus'	13±10 A ab*	27±21 A a	18±20 A ab	30±21 A a	1.4±0.5 A ab	**1.7±0.3** A a	1.3±0.3 A a	1.6±0.2 A ab
'Terratop'	31±14 A b	**45±27** A a	25±18 A b	**47±31** A a	1.4±0.2 A ab	1.4±0.1 A a	1.3±0.4 A a	2.0±0.4 B ab
'Terrafit'	20±9 A ab	31±26 A a	20±21 A ab	35±27 A a	1.4±0.1 AB ab	1.1±0.2 A a	1.3±0.2 AB a	1.8±0.4 B ab
'Energy'	**42±23** B b	22±20 AB a	20±28 A a	42±17 B a	1.5±0.2 A ab	1.3±0.2 A a	1.4±0.4 A a	1.6±0.3 A ab
SFB18/1	14±15 A a	21±15 B a	16±15 AB ab	30±23 B a	1.0±0 A a	1.5±0.3 AB a	1.1±0.2 A a	**2.1±0.7** B b
SFB22/15	22±11 A ab	24±17 A a	36±36 A b	35±18 A a	1.4±0.3 A ab	1.3±0.2 A a	1.4±0.1 A a	1.4±0.2 A a
CR2649	38±17 A b	35±23 A a	27±16 A b	40±16 A a	**1.9±0.5** B b	1.6±0.2 AB a	1.3±0.3 A a	1.5±0.2 AB ab
CR2664	30±14 A b	38±30 A a	**40±27** A b	46±19 A a	**1.9±0.3** A b	1.6±0.1 A a	**1.7±0.2** A a	1.8±0.1 A ab

Deviance analysis	
Factor	P
Repetition	0.0667
Medium	0.0002 ***
Line	4.2 e-05***
Repetition x Medium	0.8843
Repetition x Line	0.6134
Medium x Line	0.4893
Repetition x Medium x Line	0.002774 **

Deviance analysis	
Factor	p
Repetition	0.1976
Medium	9.0 e-05***
Line	0.0426 *
Medium x Line	0.0065 **

*Capital letters indicate comparisons among media within one line, whereas small letters indicate comparisons among lines within one medium. Values sharing the same letter did not differ significantly (Tukey-test *P<0.05*). The maximal values for each medium are indicated by bold letters, the maximal values for each line are highlighted by grey cells.

Regeneration rates of 40-45% were reached, but only for single lines on medium SIM 1 (2.22 µM BAP and 0.49 µM IBA), SIM 2 (4.44 µM BAP and 0.49 µM IBA) and SIM 3 (8.88 µM BAP + 0.49 µM IBA), whereas in other lines significantly less explants formed shoots. Due to high standard deviations, the different reactions of the lines were often not significant within one medium. Overall, the highest shoot regeneration rates were observed for lines 'Terratop' and CR2664.

Regarding the mean number of shoots per regenerating explant, again the medium SIM 4 presented the highest shoot numbers per regenerating explant of 2.1 and 2 shoots for the European lines SFB18/1 and 'Terratop', respectively, while on medium SIM 1, the highest number of 1.9 shoots was observed for both Indian lines CR2649 and CR2664.

Experiment 2: Testing lower BAP concentrations and silver nitrate

Since the overall shoot regeneration rate in experiment 1 of less than 50% and the number of shoots obtained per explant were rather low, further optimization was pursued in experiment 2 in which the concentration of BAP was significantly reduced in order to preserve the identity of the regenerated shoots. Moreover, based on the publication of Pua and Chi, (1993) the effect of $AgNO_3$ on the adventitious shoot regeneration was as well tested. Thus, three media: SIM 4 (best medium from experiment 1), SIM 5 and SIM 6 (both containing $AgNO_3$) (Table 1) were tested using cotyledon explants of five brown mustard lines (three European lines: 'Terratop', 'Energy' and SFB22/15 and two Indian lines: CR2649 and CR2664) (Table 3).

Table 3. Effects of different plant growth regulator combinations and silver nitrate ($AgNO_3$) on adventitious shoot regeneration from cotyledon explants of five lines of *Brassica juncea* (three European lines: 'Terratop', 'Energy' and SFB22/15 and two Indian lines: CR2649 and CR2664) (Experiment 2). MS salts and vitamins were used in all media and explants were cultivated for 4 weeks on the different media. SIM 4: 17.76 µM BAP + 4.92 µM IBA, SIM 5: 8.88 µM BAP + 5.37 µM NAA+ 9.95 µM $AgNO_3$, SIM 6: 8.8 µM BAP + 0.49 µM IBA+ 9.95 µM $AgNO_3$. The replicate numbers are specified in Supplemental Table S2.

	Shoot regeneration (%)			Mean number of shoots per regenerating explant		
Line	SIM 4	SIM 5	SIM 6	SIM 4	SIM 5	SIM 6
'Terratop'	38±17 A ab*	72±18 B a	**65±23** B ab	**2.4±0.1** B b	2.5±0.6 B ab	1.7±0.1 A a
'Energy'	29±14 A a	58±25 B a	40±23 AB a	2.0±0.4 AB ab	2.2±0.1 B a	1.7±0.2 A ab
SFB22/15	37±25 A a	70±22 B a	40±25 A ab	1.8±0.4 A ab	2.7±0.3 B ab	2.0±0.3 A abc
CR2649	49±23 A ab	65±27 A a	**65±18** A ab	1.8±0.3 A a	2.3±0.4 B a	2.1±0.2 B c
CR2664	**65±17** A b	**90±10** A a	**65±20** A b	1.8±0.1 A ab	**3.0±0.2** B b	**2.2±0.2** A bc

Deviance analysis		Deviance analysis	
Factor	p	Factor	p
Repetition	0.578	Repetition	0.4068
Medium	1.282 e-10 ***	Medium	3.165 e-12 ***
Line	1.34 e-8 ***	Line	0.00635 **
Repetition x Medium	0.5811	Medium x Line	7.82 e-05 ***
Medium x Line	0.0162 *		
Repetition x Medium x Line	0.8372		

*Capital letters indicate comparisons among media within one line, whereas small letters indicate comparisons among lines within one medium. Values sharing the same letter did not differ significantly (Tukey-test *P<0.05*). The maximal values for each medium are indicated by bold letters, the maximal values for each line are highlighted by grey cells.

The frequency of shoot regeneration and the number of shoots per regenerating explant were significantly enhanced when explants were cultured on the media SIM 5 and SIM 6 (Table 3, Figure 1). Medium SIM 5 resulted in the highest regeneration rates for all lines with the maximum of 90% of explants forming shoots in line CR2664. Not only the percentage of

regenerating explants, but also the number of shoots formed per explant were improved for all lines. On the superior medium SIM 5; 2.2 to 3 shoots per shoot forming explant were recorded.

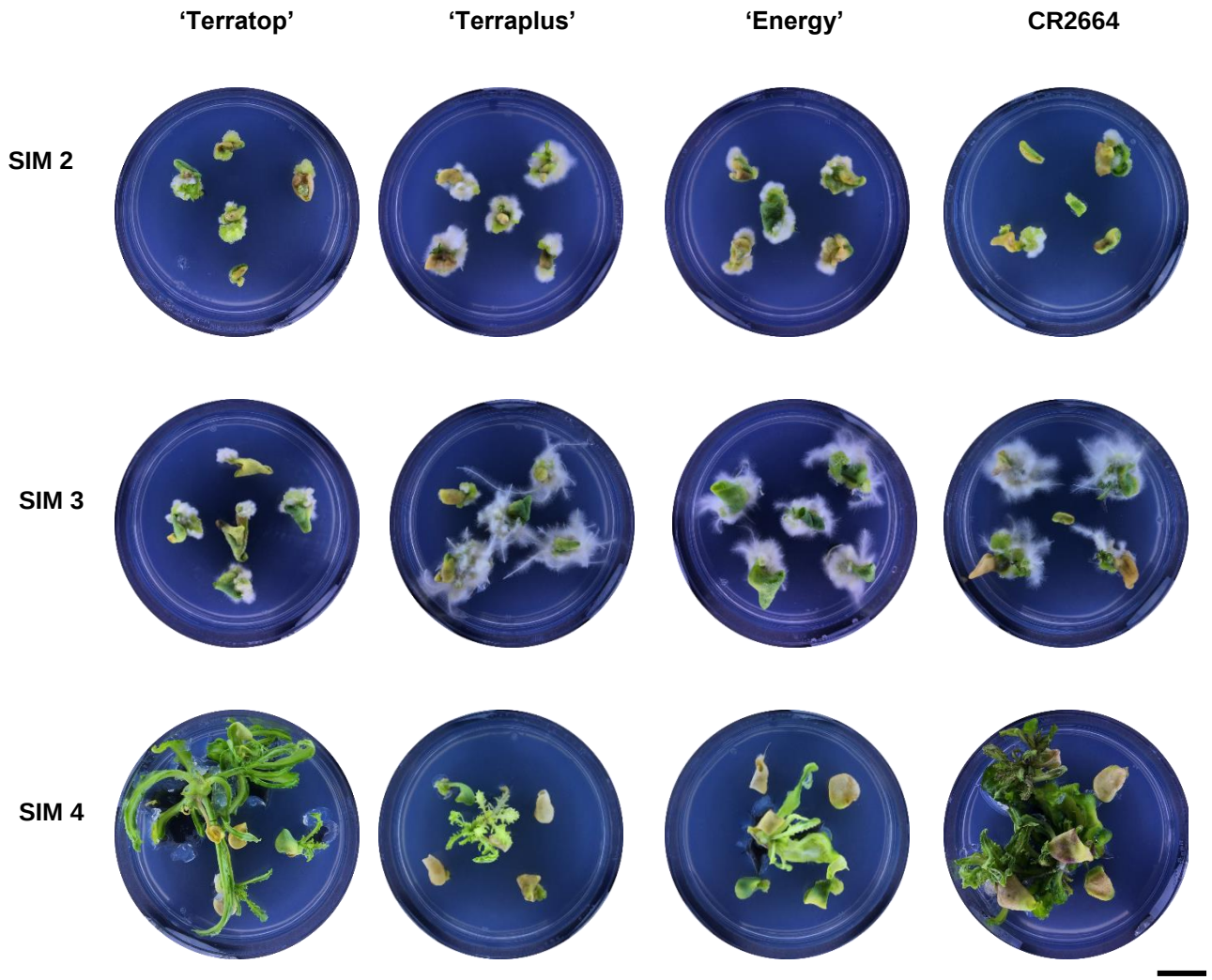

Figure 1. Effect of plant growth regulators on adventitious shoot regeneration from cotyledon explants of four lines of *Brassica juncea* (three European lines: 'Terratop', 'Terraplus', 'Energy' and an Indian line CR2664). MS salts and vitamins were used in all media and explants were cultivated for 4 weeks on the different media SIM 2: 4.44 µM BAP + 0.49 µM IBA, SIM 3: 8.88 µM BAP + 0.49 µM IBA and SIM 4: 17.76 µM BAP + 0.49µM IBA). Bar = 1 cm

Experiment 3: Effect of ethylene on adventitious regeneration

The ethylene concentration in the headspace above the explants and the effect of ethylene on shoot regeneration were further investigated by the application of ACC, an immediate ethylene precursor and $AgNO_3$. Ethylene production of the cotyledon explants of all lines was relatively low in the first two days after culture induction (Fig. 2).

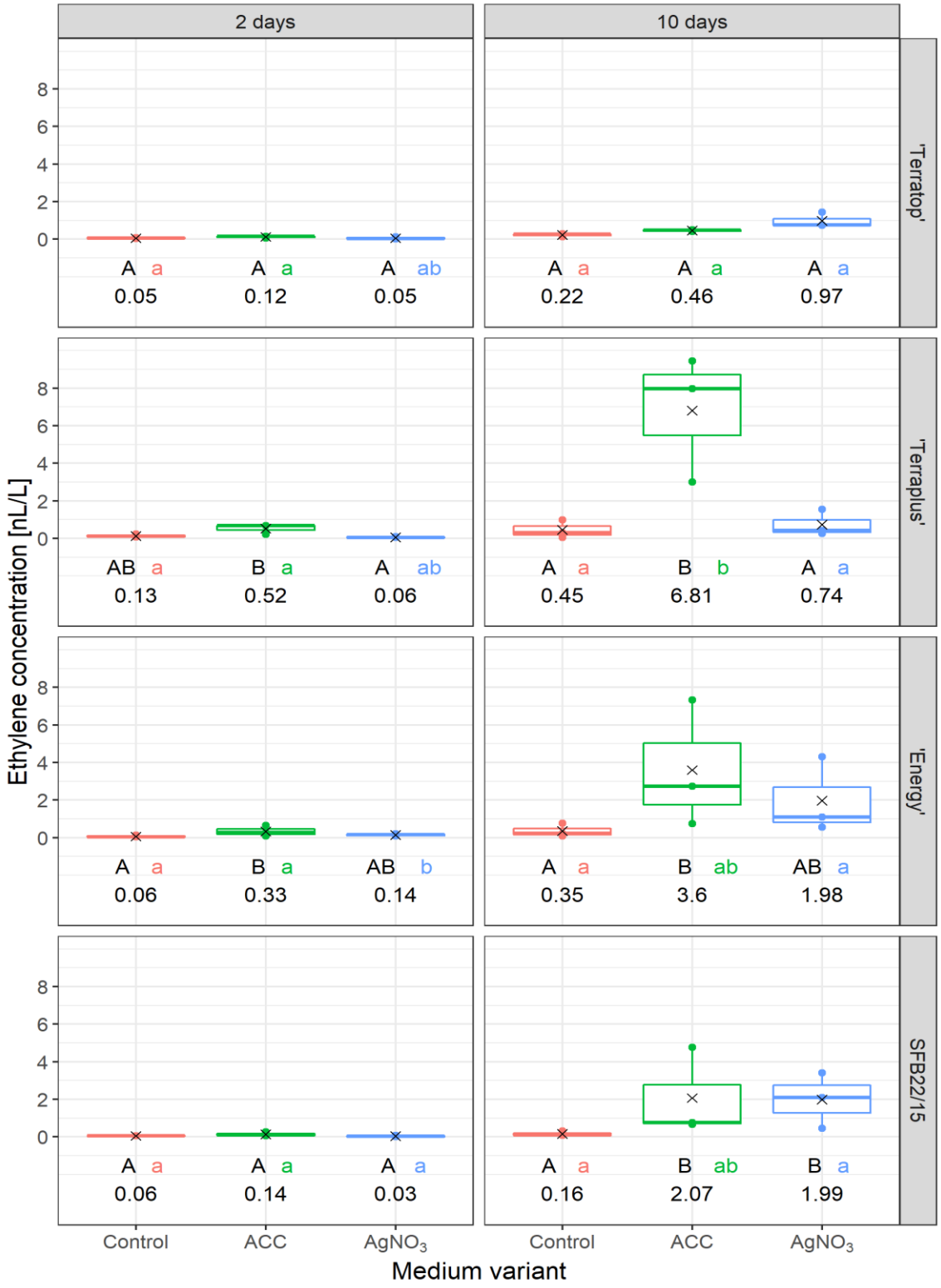

Figure 2. Ethylene production after 2 and 10 days of culture by the cotyledon explants of four European lines of *Brassica juncea* (lines: 'Terratop', 'Terraplus', 'Energy' and SFB22/15) in response to the ethylene precursor ACC and the ethylene action inhibitor $AgNO_3$. Control: 8.88 µM BAP + 5.35 µM NAA, ACC: 8.88 µM BAP + 5.35 µM NAA + 500 µM ACC and $AgNO_3$(SIM 5): 8.88 µM BAP + 5.37 µM NAA + 9.95 µM AgNO3. For each treatment, 3 Petri dish replicates containing each 8 explants were used and ethylene was measured after 2 and 10 days. Capital letters indicate comparisons among media within one line, whereas small letters indicate comparisons among lines within one medium. Values sharing the same letter did not differ significantly (Tukey-test *P<0.05*).

Nevertheless, compared to the control, on the medium with ACC, slightly elevated ethylene concentrations of 0.60 nL L^{-1} and 0.40 nL L^{-1} were measured for 'Terraplus' and 'Energy', respectively. No significant differences in ethylene concentrations were recorded between the control and the $AgNO_3$ (SIM 5) containing medium. An increase in ethylene production was observed 10 days after the culture initiation. Again, the highest ethylene production was found in the medium variant ACC and the lines 'Terraplus' and 'Energy' with 6.81 nL L^{-1} and 3.60 nL L^{-1}. At this timepoint, also explants cultivated on $AgNO_3$ (SIM 5) produced ethylene in considerable amounts, especially those of SFB22/15 and 'Energy' with 1.99 nL L^{-1} and 1.98 nL L^{-1}, respectively. The lowest ethylene production was observed for explants on control medium. When comparing the lines, 'Terratop' stood out due to a low ethylene production, even on ACC medium.

Regarding the effect of ethylene on shoot regeneration, fourteen days after culture induction, most of the cotyledon explants of all four lines cultured on the medium variants ACC progressively turned yellow and the explant growth and adventitious shoot formation were clearly higher on $AgNO_3$ containing medium (Supplemental Figure S4). The ACC containing medium led to the highest ethylene production and at the same time the lowest shoot regeneration rates between 1% and 9.8% (Table 4). The control variants showed shoot regeneration in 10.8% to 55.8%, whereas on medium with $AgNO_3$ significantly higher regeneration rates ranging from 49.2% to 77.3% were observed. Among the lines, 'Terratop' and 'Terraplus' were superior in both, shoot regeneration rate and number of shoots formed (Table 4).

Table 4. Effect of ACC and $AgNO_3$ on adventitious regeneration from cotyledon explants of four European lines of *Brassica juncea* ('Terratop', 'Terraplus', 'Energy' and SFB22/15) (Experiment 3). MS salts and vitamins were used in all media variants and explants were cultivated for 4 weeks on the different media. Control: 8.88 µM BAP + 5.37µM NAA, ACC: 8.88 µM BAP + 5.37µM NAA + 500 µM ACC, $AgNO_3$ (SIM 5): 8.88 µM BAP + 5.37µM NAA + 9.95 µM $AgNO_3$.The replicate numbers are specified in Supplemental Table S3.

	Shoot regeneration (%)			Mean number of shoots per regenerating explant		
Line	Control	ACC	$AgNO_3$	Control	ACC	$AgNO_3$
'Terratop'	**56±22** B b*	5±9 A a	**77±10** C b	**1.4±0.5** A a	**1.7±0.6** AB a	**2.0±0.8** B b
'Terraplus'	11±15 B a	1±4 A a	76±30 C b	**1.4±0.4** A a	1.0±0 A a	**2.0±0.7** A ab
'Energy'	16±19 A a	**10±14** A a	67±26 B ab	1.2±0.4 A a	1.2±0.4 A a	1.7±0.6 A ab
SFB22/15	23±17 B a	7±10 A a	49±22 C a	1.2±0.6 A a	1.3±0.5 A a	1.6±0.6 A a

Deviance analysis		Deviance analysis	
Factor	p	Factor	p
Medium	2.2 e-16 ***	Medium	2.218 e-07 ***
Line	9.95 e-05 ***	Line	0.005268 **
Medium x Line	0.000303 ***	Medium x Line	0.9654

*Capital letters indicate comparisons among variants within one line, whereas small letters indicate comparisons among lines within one variant. Values sharing the same letter did not differ significantly (Tukey-test *P<0.05*). The maximal values for each variant are indicated by bold letters, the maximal values for each line are highlighted by grey cells.

While ACC resulted in chlorotic explants with only small amounts of callus and some adventitious roots, shoot regeneration and also shoot growth of all four European lines was greatly improved by the addition of $AgNO_3$ (Figure 3).

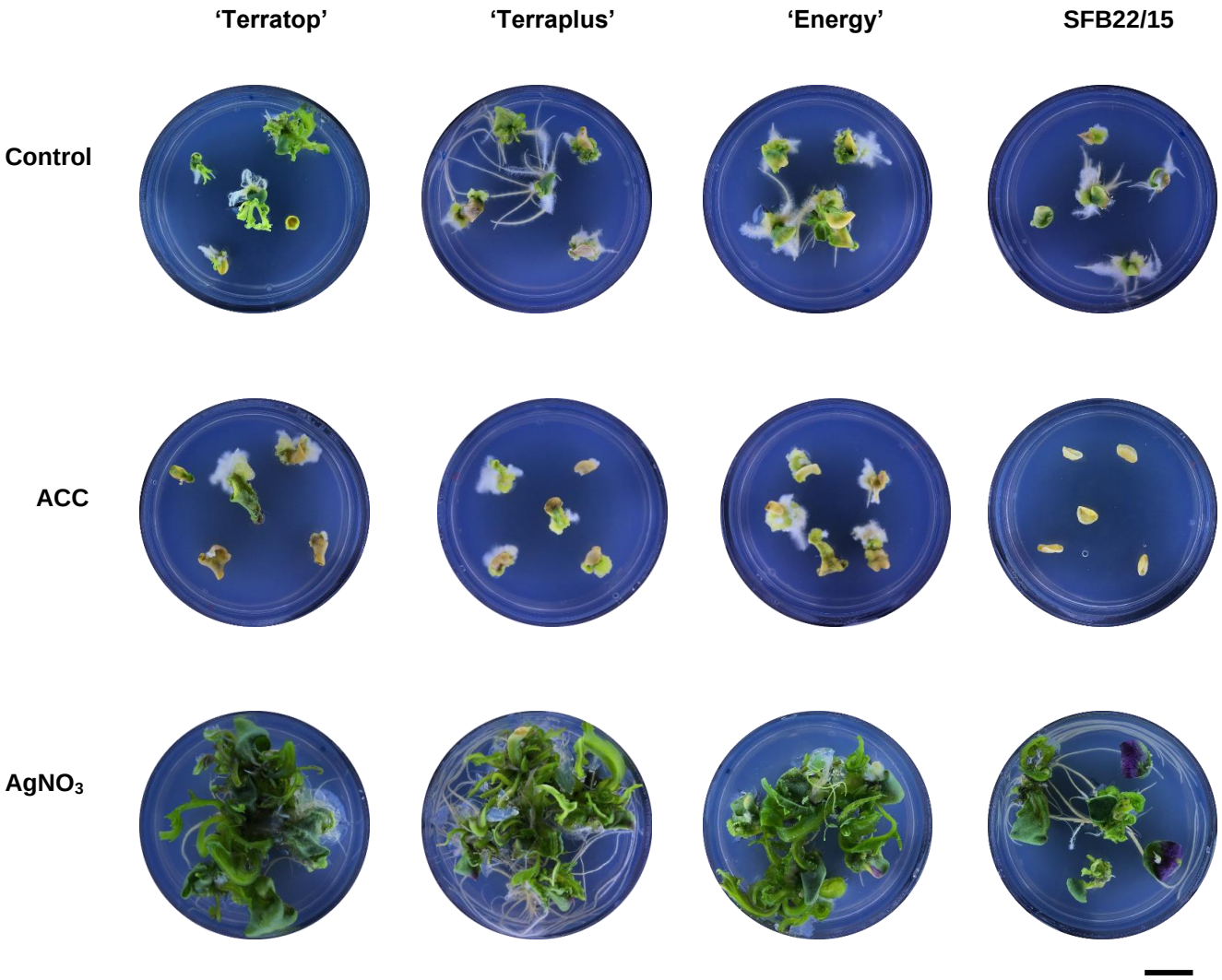

Figure 3. Effect of the ethylene precursor ACC and the ethylene action blocker $AgNO_3$ on adventitious regeneration from cotyledon explants of four European lines of *Brassica juncea* ('Terratop', 'Terraplus', 'Energy' and SFB22/15). MS salts and vitamins were used in all media variants and explants were cultivated for 4 weeks on the different media. Control: 8.88 µM BAP + 5.37µM NAA, ACC: 8.88 µM BAP + 5.37µM NAA + 500 µM ACC, $AgNO_3$ (SIM 5): 8.88 µM BAP + 5.37µM NAA + 9.95 µM $AgNO_3$. Bar = 1 cm.

4 Discussion

4.1 Effects of plant growth regulators, explant type and line

In this study, the conditions for efficient adventitious shoot regeneration in different European *B. juncea* lines were studied. We focused on six European lines ('Terraplus', 'Terratop', 'Terrafit', 'Energy', SFB18/1 and SFB22/15) obtained from a German breeder and mainly used as catch crop and two Indian lines (CR2649 and CR2664) from a gene bank. The first experiment showed firstly, that less than 5% of the hypocotyl explants formed shoots, which

may have originated from remaining apical meristem and therefore, were not considered to be true adventitious shoots. Also, George and Rao (1980) reported cotyledons to be more suitable explants for shoot regeneration of the Indian line *B. juncea* var. Rai-5 with a regeneration rate of 66% for cotyledon explants against 0% for hypocotyl explants cultured on medium supplemented with 4.44 µM BAP and 5.37 µM NAA. Since different explant types in *Brassica* responded differently to particular combinations of auxin and cytokinin (Lazzeri et al., 1986), for hypocotyls other plant growth regulator combinations could be tested in the future. Likewise, the adventitious shoot regeneration from hypocotyl explants could be markedly improved by replacing agar with 0.4% agarose (Pua, 1994). Nevertheless, due to their clearly better response, cotyledons were used in all our subsequent experiments and their response will be discussed in the following.

The second outcome of the first experiment was that the regeneration rates of European and Indian lines were quite similar ranging between 30-47% for the European lines and 40-46% for the Indian lines for the best medium SIM 4 containing high concentrations of BAP (17.76 µM) and the IBA (4.92 µM) (Table 2). However, none of the tested combinations of BAP and IBA could induce a frequency of more than 50% shoot regeneration, and also, the number of shoots produced per explant was low. Fazekas et al. (1986) observed for cotyledon explants of 8 Indian and 13 European lines cultured on medium containing 4.44 µM BAP and 5.37 µM NAA regeneration rates of 20% to 50% for the Indian lines against 0% to 12% for the European lines with an average of 2 shoots per regenerating explant.

Besides a different requirement of exogenous plant growth regulators among lines, the variability and poor regeneration might be attributed to the presence of factors of the *B. campestris* genome (Narasimhulu and Chopra, 1988), because this parental species and donor of the A genome has a low regeneration ability. One factor contributing to poor in vitro regeneration responses of *Brassica* can be ethylene (Pua and Chi, 1993), and thus, we tested a medium (SIM 5) containing the ethylene receptor inhibitor $AgNO_3$ for a selection of *B. juncea* lines in experiment 2.

4.2 Improved shoot regeneration in both Indian and European lines, on a medium containing silver nitrate

The use of medium SIM 5 containing $AgNO_3$ significantly enhanced the shoot regeneration of both, European and Indian lines of *B. juncea* (Table 3, Table 4). This significant improvement in the regeneration frequency of the different lines compared to the best medium of experiment 1 did not only result from the use of $AgNO_3$, but may also be partly due to the different combination of auxin and cytokinin. For all six lines tested, the combination of 8.88 µM BAP and 5.37 µM NAA and 9.95 µM $AgNO_3$, in the medium SIM 5 resulted the highest regeneration frequencies (Table 3). This growth regulator combination was also recommended in previous studies involving other lines of *B. juncea* (Barfield and Pua, 1991; Pua and Chi, 1993; Pua et al., 1999; Bhuiyan et al., 2009a).

The complementary action of $AgNO_3$ and the plant growth regulator combination (BAP and NAA) used in SIM 5 was not only observed for the frequency of shoot regeneration which increased to 58-90%, but also for the number of regenerants per explant (2.2-3 shoots per regenerating explant on medium SIM 5, Table 3). Several reports attributed an improvement of the shoot regeneration frequency to the promotive effect of the ethylene physiological action inhibitor $AgNO_3$ on shoot regeneration of variety of *Brassica* species. For instance, the recalcitrant parental species *B. campestris* (shoot regeneration frequency increased from 6.1% to 83.6% as well as the number of regenerants per explant from 3.2 to 11.4 shoots using 60 µM $AgNO_3$ (Palmer, 1992), but also *B. oleracea* (20 µM $AgNO_3$ enhanced the shoot regeneration frequency from 30% to 90% (Pua et al., 1993).

The positive effect of $AgNO_3$, is due to the silver ion (Ag^+) which blocks plant ethylene responses (Beyer, 1976) by binding to ethylene receptors of the subfamily I, mainly ETR1, and inhibiting their action (Binder, 2020). The fact that the application of the receptor inhibitor $AgNO_3$ had a positive effect on shoot regeneration suggested that ethylene itself might have a negative effect on this process. To further investigate whether ethylene has an effect on

adventitious shoot formation in *B. juncea*, we carried out experiment 3, in which we applied the ethylene precursor ACC.

4.3 Ethylene inhibited shoot regeneration

Feeding the precursor ACC, indeed resulted in increased ethylene production by the cotyledon explants already after two days of culture, but more pronounced after ten days (Figure 2). This was the case for three of four European lines tested ('Terraplus', 'Energy' and SFB22/15), but not for 'Terratop'. Pua and Chi (1993) measured the ethylene production of leaf explants of the Indian lines of *B. juncea*, fourteen days after culture induction ranging from (0.19 to 0.67 µL L^{-1} h^{-1}) resulting in poor regeneration frequencies (20%-30%). Since the Petri dishes used in our study were only sealed with Parafilm and therefore not tight enough to allow accurate determination of ethylene production rates as done in the study of Pua and Chi (1993), the ethylene levels cannot be compared to their data. The setup was only meant to compare among lines and treatments. Besides the strikingly different reaction of 'Terratop' the second important insight was gained from the comparison of the control variant and the $AgNO_3$ variant. Cotyledon explants cultivated on $AgNO_3$ containing medium produced higher amounts of ethylene after 10 days of culture than untreated control explants. This observation is in accordance with previous reports on the inhibition of the ethylene receptor triggering cells to produce ethylene (Theologis, 1992).

Shoot regeneration was severely affected in all lines by the ethylene produced by the cotyledon explants cultured on ACC-containing medium. Furthermore, chlorosis, necrosis, strong root hair formation and a reduced expansion of the cotyledon explants was recorded (Figure 3). Similar results were reported by Jha et al. (2007) when evaluating the ethylene effect on the plant regeneration of six genotypes of barley. Interestingly, the regeneration rate was significantly reduced in most lines (< 30%), only 'Terratop' seemed to be less sensitive to ethylene with a regeneration rate of 56% and for this line lowest ethylene concentrations were observed after 10 days. Ethylene production and action is highly regulated, and might affect

organogenesis in concentrations as little as 0.01 µL L^{-1} (Reid 1995). Thus, the concentrations measured in our experiment are physiologically relevant.

Also, in experiment 3, the promotive effect of the ethylene action inhibitor $AgNO_3$ on the organogenesis of *B. juncea* was seen. Although ethylene was produced in higher amounts by the cotyledon explants cultured on the medium with $AgNO_3$ (3 to 12-fold increase compared to the control), the regeneration rate was significantly enhanced (21% to 65%) in all *B. juncea* lines pointing to a negative effect of ethylene especially in the early stages of organogenesis. The improvement of the in vitro shoot regeneration efficiency by the use of the ethylene inhibitor $AgNO_3$ to prevent the ethylene action on the cotyledon explants of *B. juncea* in this study is in line with previous reports (Barfield and Pua, 1991; Pua and Chi, 1993; Pua et al., 1999; Bhuiyan et al., 2009a).

But the use of $AgNO_3$ can also lead to growth inhibition in plants. Pua and Chi (1993) found an inhibitory effect of $AgNO_3$ on *B. juncea*, if applied four weeks after the culture induction, with 61% decrease in plant fresh weight and 75% in plant height, resulting in fewer and shorter leaves and roots. Thus, it should be tested in future experiment, for how long the explants should best be cultured on $AgNO_3$ containing medium.

Although silver nitrate is a commonly used source of Ag^+ to influence the regeneration potential in a variety of plants, silver thiosulfate was also applied successfully to improve shoot regeneration in *Brassica* species (Eapen and George, 1996; Eapen and George, 1997). Moreover, the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) may be another option, since it does not lead to an overproduction of ethylene that was observed for $AgNO_3$ (Pua and Chi, 1993).

5 Conclusion

The identification of the appropriate conditions for efficient adventitious shoot regeneration of European *B. juncea* lines is useful for establishing a genetic transformation system to develop improved *B. juncea* lines using genome editing techniques. The combination of 8.88 µM BAP and 5.37 µM NAA and 9.95 µM $AgNO_3$ gave the highest adventitious regeneration rates. The use of silver nitrate has a stimulatory effect on the shoot organogenesis of *B. juncea*. Furthermore, one major factor limiting shoot regeneration of *B. juncea* was the ethylene production which significantly affects the early events during shoot organogenesis responses of the cultured tissues. Since ethylene is involved in the regulation of several developmental processes, future studies should identify time windows in which ethylene action blocking by $AgNO_3$ is most effective.

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3 Removing the major allergen Bra J I from brown mustard (*Brassica juncea*) by CRISPR/Cas9

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Traud Winkelmann	Conceived and designed the experiments, coordinated the research, contributed to the writing of the manuscript
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SUMMARY

Food allergies are a major health issue worldwide. Modern breeding techniques such as genome editing via CRISPR/Cas9 have the potential to mitigate this by targeting allergens in plants. This study addressed the major allergen Bra j I, a seed storage protein of the 2S albumin class, in the allotetraploid brown mustard (*Brassica juncea*). Cotyledon explants of an Indian gene bank accession (CR2664) and the German variety Terratop were transformed using *Agrobacterium tumefaciens* harboring binary vectors with multiple single guide RNAs to induce either large deletions or frameshift mutations in both *Bra j I* homoeologs. A total of 49 T_0 lines were obtained with up to 3.8% transformation efficiency. Four lines had large deletions of 566 up to 790 bp in the *Bra j IB* allele. Among 18 Terratop T_0 lines, nine carried indels in the targeted regions. From 16 analyzed CR2664 T_0 lines, 14 held indels and three had all four *Bra j I* alleles mutated. The majority of the CRISPR/Cas9-induced mutations were heritable to T_1 progenies. In some edited lines, seed formation and viability were reduced and seeds showed a precocious development of the embryo leading to a rupture of the testa already in the siliques. Immunoblotting using newly developed Bra j I-specific antibodies revealed the amount of Bra j I protein to be reduced or absent in seed extracts of selected lines. Removing an allergenic determinant from mustard is an important first step towards the development of safer food crops.

Keywords: mustard, Bra j I, CRISPR/Cas, transformation, food allergen, seed storage protein.

INTRODUCTION

Food allergy is considered a public health issue and the prevalence has risen in recent decades. In sensitized persons, food allergy can elicit allergic symptoms such as urticaria, itching, wheezing, dyspnea, abdominal pain, and live-threatening anaphylactic shocks (Matsuo et al., 2015). Plant food allergenic proteins are classified according to their structural or functional properties. Most of these belong to the cupin superfamily (7S and 11S seed storage proteins) or the prolamin superfamily (2S albumins, nonspecific lipid transfer proteins, α-amylase/trypsin inhibitors, cereal prolamins) or are functional plant defense proteins (pathogenesis-related proteins, proteases, and protease inhibitors) (Breiteneder and Ebner, 2000; Breiteneder and Radauer, 2004). Most of the 2S albumins are heterodimeric proteins consisting of a large and a small subunit that are connected by a disulfide bond (Shewry et al., 1995).

The known allergens among the 2S albumins include Sin a I from yellow mustard seeds (*Sinapis alba*) (Menéndez-Arias et al., 1988), Bra j I from oriental or brown mustard seeds (*Brassica juncea*) (González de la Peña et al., 1991; Monsalve et al., 1993), Ber e 1 from Brazil nut (*Bertholletia excelsior*) (Nordlee et al., 1996), Jug r 1 from the English walnut (*Juglans regia*) (Teuber et al., 1998), and Ses i 2 from sesame (*Sesamum indicum*) (Beyer et al., 2002). Mustard is a significant elicitor of allergic reactions to food, with yellow and black mustard being mostly consumed in Europe, whereas the brown mustard flour (*B. juncea* L.) is most commonly used in mustard extracts in the USA and Japan (González de la Peña et al., 1991; Monsalve et al., 2001). Mustard allergy usually appears before the age of three, thus mustard is considered as a hidden allergen in the infant's diet (Rancé et al., 2000). Currently, the most effective treatment of mustard and other food

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allergies is to strictly avoid ingesting allergen-containing food. However, accidental consumption of allergen-containing food is a constant issue and sensitized persons are significantly compromised in their daily life. Therefore, the development of allergen-free foods is a strategy that would make foods safe for sensitized persons and relieve them from a serious burden.

Using random mutagenesis or traditional breeding strategies to obtain allergen-free plants is difficult and time consuming, especially for polyploid crops with multiple gene copies. Genetic engineering technologies such as RNA interference (RNAi) have been used to alleviate peanut (*Arachis hypogaea*) allergy by silencing genes encoding allergens (Chandran et al., 2015; Dodo et al., 2008). Using programmable nucleases, like CRISPR/Cas9 or TALEN, targeted random mutations and deletions can be induced (Manghwar et al., 2019). This breeding method is currently the one with the greatest potential for crop improvements (Gao, 2021). Many countries have classified genome-edited crops as non-genetically modified organisms, relieving them from extensive regulation, and the first products from genome-edited plants have reached the market (Metje-Sprink et al., 2020). As a first example for a low-allergen food, CRISPR/Cas9 has been used to create low-gluten wheat (*Triticum aestivum*) for gluten-intolerant consumers (Sánchez-León et al., 2018).

Brassica juncea (L.) Czern is one of the major oilseed brassicas, and for this purpose it is mostly cultivated in semi-arid tropics of the Indian subcontinent, as well as in drier areas of Canada, Australia, China, Russia, and the northern USA (Labana and Gupta, 1993). *Brassica juncea* is a self-pollinated plant and an amphidiploid (allotetraploid) hybrid (AABB; 2n = 36), derived from the two diploid progenitors *Brassica rapa* (AA genome, 2n = 20) and *Brassica nigra* (BB genome, 2n = 16) followed by successive chromosome doubling thousands of years ago (Redden et al., 2009; Yang et al., 2016). *Brassica juncea* has been divided into four subspecies, with different morphology, quality characteristics, and usages: (i) the subspecies *B. juncea integrifolia*, used as a leafy vegetable in Asia, (ii) the subspecies *B. juncea juncea*, mostly produced for its seeds and sometimes as fodder, (iii) the subspecies *B. juncea napiformis*, mainly used as a root-tuber vegetable, and (iv) the subspecies *B. juncea taisai*, the stalks and leaves of which are used as vegetables in China (Spect and Diederichsen, 2001). The oilseed of *B. juncea* is especially rich in fatty acid and is an excellent source of natural antioxidants, but its high glucosinolate content and a fatty acid profile with a high level of erucic acid present a health issue for consumption. On the other hand, due to its high content of erucic acid with its low flash points and good combustion and lubrication qualities it can also be a valuable component in biodiesel (Premi et al., 2013). Moreover, the high glucosinolate content renders *B. juncea* an excellent product for biofumigation as a tool to alleviate the effects of soilborne pathogens and replant diseases (Hanschen and Winkelmann, 2020; Mattner et al., 2008; Yim et al., 2016).

To date, no study has reported the production of agronomically viable low-allergenic lines in *B. juncea*. However, several studies have reported the use of genetic engineering to develop varieties of *B. juncea* improved in oil and meal quality. Augustine et al. (2013) applied RNAi-based target suppression to develop low-glucosinolate *B. juncea* lines with significantly improved seed meal quality. Furthermore, Sinha et al. (2007) reported using hairpin-RNA to silence the *fatty acid elongase* gene in *B. juncea*, resulting in a decrease of erucic acid production. Yusuf and Sarin (2007) were successful in using genetic engineering methods to overexpress the γ-*TMT* gene to increase the vitamin E (α-tocopherol) level in the seeds of *B. juncea*.

To our knowledge, the use of CRISPR/Cas9 in brown mustard has not been reported yet. Here, we show that CRISPR/Cas9 can be used to precisely and efficiently mutate the *Bra j I* gene in this allotetraploid crop such that the allergenic Bra j I protein is absent from mutant seeds.

RESULTS

Design of CRISPR/Cas9 constructs with multiplex sgRNA expression cassettes targeting *Bra j I*

Two brown mustard (*B. juncea*) lines were chosen for this study, a European (Terratop) and an Indian (CR2664) one. The two *Bra j I* homoeologs in the published genome of brown mustard (*Bra j IA* and *Bra j IB*) have highly similar nucleotide sequences and are identical for both mustard lines (Figure S1). To modify the two *Bra j I* homoeologs, we designed eight single guide RNAs (sgRNAs) (sg1, sg2, sg3, sg4, sg5, sg6, sg7A, and sg7B) targeting conserved regions in the *Bra j I* exon or 5′/3′-untranslated region (UTR) regions (Figure 1a, Figure S1, and Table S1). All sgRNAs were individually driven by the *AtU6* promoter. Two final binary constructs (named pBraj1256 and pBraj3477) both carrying genes for *SpCas9*, hygromycin resistance, and sgRNA expression cassettes were generated (Figure 1b). pBraj1256 harbored four sgRNAs (sg1, sg2, sg5, and sg6) and could potentially lead to a complete deletion of *Bra j IA* and *Bra j IB* by simultaneously targeting their 5′- and 3′-UTR regions. pBraj3477 contained four sgRNAs (sg3, sg4, sg7A, and sg7B) targeting *Bra j I* coding sequences to introduce deletions or insertions at the cleavage sites that can lead to frameshift mutations of *Bra j IA* and *Bra j IB*.

Genetic transformation of mustard

Cotyledon explants of the *B. juncea* lines Terratop and CR2664 were transformed with the binary vectors containing either the reporter gene construct pEGFP (carrying an

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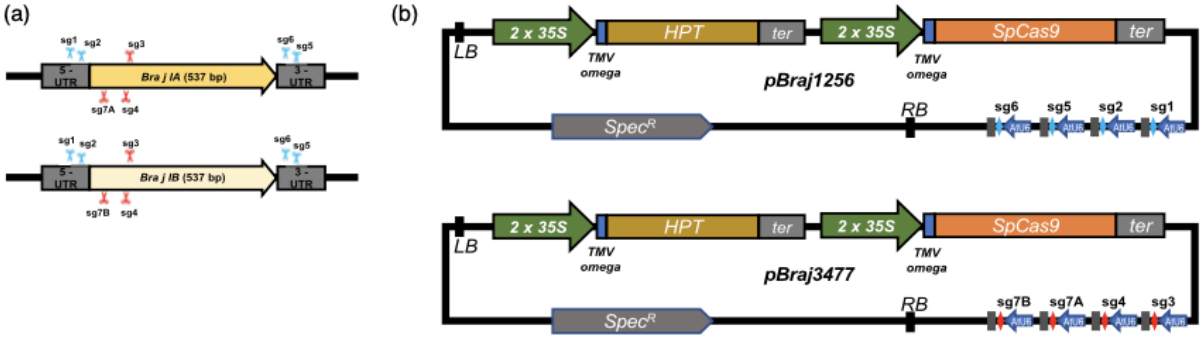

Figure 1. Constructs for targeted mutagenesis of *Bra j I* in *B. juncea* using CRISPR/Cas9. (a) Cartoon of the homoeologous *Bra j I* genes in the A and B subgenomes of tetraploid brown mustard (*B. juncea*) with sgRNA target sites (scissors). The four sgRNAs sg1, sg2, sg5, and sg6 (blue) target conserved 5′- and 3′-untranslated regions of *Bra j IA* and *Bra j IB*, whereas the four sgRNAs sg3, sg4, sg7A, and sg7B (red) target the *Bra j IA* and *Bra j IB* coding sequences. (b) Schematic of binary vectors designed for deleting (pBraj1256) or disrupting (pBraj3477) the coding sequences of *Bra j IA* and *Bra j IB*, respectively. *HPT*, *SpCas9*, and *Spec*^{R} encode hygromycin B phosphotransferase, *Streptococcus pyogenes* Cas9 endonuclease, and a spectinomycin resistance protein, respectively.

enhanced green fluorescence protein-encoding gene and a kanamycin selection marker) or one of two CRISPR/Cas9 constructs (pBraj1256 and pBraj3477, carrying a hygromycin selection marker) (Figure 2a,b). The regeneration of first putative transgenic shoots was observed 4 weeks after infection with *A. tumefaciens* (Figure 2c) and during the following three culture passages developed into plantlets (Figure 2d). Within the first 3 months, some weaker shoots died and these non-transgenic escapes were characterized by an albino-like phenotype. Only the shoots that survived after four culture passages on selection medium were considered to be transgenic shoots and were further tested by PCR. The pEGFP construct was used to establish the genetic transformation protocol (Table 1). To characterize the 57 putative transgenic plants obtained from the two CRISPR/Cas9 constructs, three vector-specific primer pairs targeting different regions of the transfer DNA (T-DNA) were used. Based on the PCR results, 19 out of 23 Terratop T_0 plants and 30 out of 34 CR2664 T_0 plants were PCR-positive plants (Figure S2). The transformation efficiency was line-dependent and also varied with the binary vectors used (Table 1) as well as among the independent experiments (Table S1). The transformation efficiencies in the line Terratop were 2 and 3.8% for pBraj1256 and pBraj3477, respectively, whereas in the line CR2664, transformation efficiencies of 3.8 and 3% were recorded with pBraj1256 and pBraj3477, respectively. In total, from the genetic transformation experiments 49 T_0 plants were transferred to the greenhouse. The surviving transgenic plants (36 T_0 plants) were grown until maturity and leaves and seeds were harvested for further characterization.

Characterization of *Bra j I* mutations in regenerated mustards

To identify CRISPR/Cas9-induced mutations in *Bra j I*, site-specific primer pairs were used to amplify the corresponding *Bra j IA* and *Bra j IB* regions from the transgenic T_0 mustard genomes. PCR on DNA from two T_0 lines (T0-1 from CR2664 and T0-47 from Terratop) which were transformed with the deletion construct pBraj1256 amplified a shorter *Bra j IB* fragment, potentially indicating a deletion (Figure S3). These DNA fragments were cloned and sequenced, revealing a 695-bp deletion of *Bra j IB* in line T0-1 (corresponding precisely to the region between the 5′- and 3′-flanking sgRNA target sites) and a 790-bp deletion of *Bra j IB* in line T0-47 (Figure S4). In addition, we found that two T_0 lines from Terratop (T0-21 and T0-55), which had been transformed with the frameshift mutation construct pBraj3477, also produced a PCR fragment indicating a deletion in *Bra j IB*. Sequencing revealed that line T0-21 has a 566-bp deletion in *Bra j IB* (the deleted region contained sg3 and sg4 target sites), and line T0-56 carries a 629-bp deletion (from the sg7 cutting site to the 3′-UTR) in *Bra j IB* (Figure S4).

Furthermore, we selected 16 transgenic T_0 lines transformed with pBraj3477 (10 from Terratop and six from CR2664) and 14 T_0 lines transformed with pBraj1256 (five from Terratop and nine from CR2664) for detailed *Bra j I* genotyping (Table 2 and Table S2). Among the 15 Terratop T_0 lines, Sanger sequencing indicated that two and five T_0 lines transformed with pBraj3477 and pBraj1256, respectively, carried indels in the targeted regions and the other eight lines did not show any mutation. For the 15 CR2664 T_0 lines, sequencing results showed that six and seven T_0 lines transformed with pBraj3477 and pBraj1256, respectively, carried indels in the targeted regions. Importantly, by specifically analyzing the *Bra j IA* and *Bra j IB* genes, we found that three CR2664 mutants (T0-22, T0-26, and T0-32) transformed with pBraj3477 had all four *Bra j I* alleles mutated (Table 2, Figure S4b).

Overall, for pBraj3477 the mutagenesis frequency of *Bra j IA*/*Bra j IB* was about 33.3 (4/12) and 100% (6/6) in

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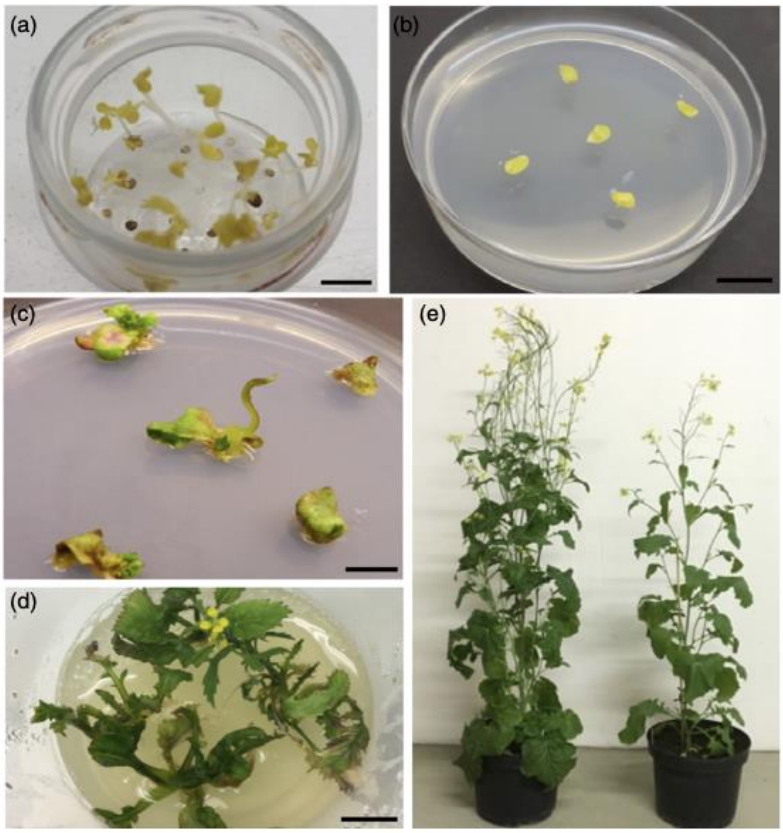

Figure 2. Generation of transgenic *B. juncea* plants (line CR2664) carrying the CRISPR/Cas9 constructs. (a) Five-day-old seedlings. (b) Cotyledon explants used for co-culture. (c) Shoot regeneration 4 weeks after co-culture with *A. tumefaciens* with pBraj1256. (d) Ten-week-old transgenic plants transformed with *A. tumefaciens* pBraj1256. (e) Flowering transgenic plants 10 weeks after acclimatization. Bars represent 1 cm.

Table 1 Transformation efficiencies for two *B. juncea* lines (Terratop and CR2664) using the three vectors pEGFP, pBraj1256, and pBraj3477

Vector[a]	Line	No. of explants[b]	No. of T_0 lines obtained	Transformation efficiency (%)[c]
pEGFP	Terratop	300	2	0.7
	CR2664	300	11	3.7
pBraj1256	Terratop	400	8	2
	CR2664	500	19	3.8
pBraj3477	Terratop	400	15	3.8
	CR2664	500	15	3

[a]Kanamycin selection for pEGFP and hygromycin selection for pBraj1256 and pBraj3477.
[b]For details see Table S1.
[c]Percentage of independent transgenic shoots per 100 explants. Transgenicity was analyzed by three different PCR reactions targeting the T-DNA.

Terratop and CR2664, respectively. Using pBraj1256, the mutagenesis frequency of *Bra j IA*/*Bra j IB* was 100 (6/6) and 80% (8/10) in Terratop and CR2664, respectively. Taken together, we successfully modified *Bra j I* in two brown mustard lines.

Inheritance of mutations in T_1 plants

To investigate the segregation of biallelic and heterozygous mutants, T_1 progenies were generated by self-pollination and analyzed. Sanger sequencing showed that 11 T_1 plants from two *bra j I* biallelic mutants (eight of them were from T0-22 and three from T0-32) were homozygous for *bra j IA* and *bra j IB* or again biallelic mutants (Table 3). For the heterozygous mutant T0-23 which harbored a biallelic mutation at the *Bra j IA* locus and a heterozygous mutation at the *Bra j IB* locus, 17 T_1 plants were analyzed, all of which were homozygous or biallelic mutations at the *Bra j IA* locus as expected. In contrast, only one offspring (line 23-7) carried a heterozygous mutation at the *Bra j IB* locus and the remaining 16 T_1 plants were homozygous wild type at the *Bra j IB* locus, which is not the expected 1:2:1 segregation ratio for a heterozygous locus after selfing (Table S3).

To assess whether the *Bra j IB* deletion identified in line T0-1 can be transmitted to the next generation, we tested 35 T_1 progeny plants, but found no PCR fragment indicating a deletion among them (Figure S5). We selected two T_1 progenies (1-1 and 1-2) from T0-1 for sequencing and new allelic mutations were detected in both *Bra j IA* and *Bra j*

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Table 2 Genetic characterization of T_0 plants of the *B. juncea* lines Terratop and CR2664 regarding transgene presence and mutations in the target genes *Bra j IA* and *Bra j IB*, seed weight, and germination of seeds

Plant number	Plasmid	Line	Transgenicity assay[a]	Genotype[b] *Bra j IA* allele 1/allele 2	Genotype[b] *Bra j IB* allele 1/allele 2	Seed number obtained from free pollination	Weight of 100 seeds [g][c]	*In vitro* germination [%] (n = 10)	*Ex vitro* germination [%] (n = 20)
K2	-	Terratop	-	Wild type/wild type	Wild type/wild type	4477	0.257	100	95
K17	-	CR2664	-	Wild type/wild type	Wild type/wild type	4490	0.289	80	95
1	pBraj1256	CR2664	+	Wild type/wild type	Wild type/−695 bp	745	0.280[ns]	40	15
22	pBraj3477	CR2664	+	−14 bp (sg7)/+1 bp (sg7)	−1 bp (sg7)/+1 bp (sg7)	119	0.095	40	55
23	pBraj3477	CR2664	+	−14 bp (sg7)/+1 bp (sg7)	Wild type/+1 bp (sg7)	65	0.218	50	85
25	pBraj1256	CR2664	+	Wild type/wild type	Wild type/wild type	307	0.103***	20	20
26	pBraj3477	CR2664	+	+1 bp (sg4), +1 bp (sg7)/+1 bp (sg4), +1 bp (sg7)	+1 bp (sg7), −4 bp (sg4)/+1 bp (sg7), −1 bp (sg4)	482	0.133***	10	30
27	pBraj1256	CR2664	+	Wild type/wild type	Wild type/wild type	125	0.068	10	20
30	pBraj1256	CR2664	+	−1bp (sg1)/−1bp (sg1)	Wild type/−8 bp (sg1)	203	0.157	50	90
31	pBraj1256	Terratop	+	−1bp (sg1)/−1bp (sg1)	−4 bp (sg1)/−1 bp (sg1)	1264	0.183***	60	85
32	pBraj3477	CR2664	+	+1 bp (sg4); +1 bp (sg7)/+1 bp (sg4); +1 bp (sg7)	+1 (sg7), +1 (sg4)/−2 (sg7)	182	0.195	0	15
33	pBraj3477	CR2664	+	Wild type/−3 bp (sg7)	Wild type/+1 bp (sg7)	1303	0.198***	0	30
34	pBraj3477	CR2664	+	Wild type/−3 bp (sg7)	Wild type/−1 bp (sg7)	155	0.163	10	0
37	pBraj1256	CR2664	+	−1 bp (sg1)/−1 bp (sg1)	+1 bp (sg1)/+1 bp (sg1)	128	0.153	80	50

[a]'+' detected, '-' not detected.
[b]The sequencing chromatograms were decoded by ICE Analysis (https://ice.synthego.com/#/).
[c]For lines with less than 300 seeds no statistical analysis was conducted. For the remaining lines the 100-seed weight of three to four subsamples was compared against the wild type by Dunnett's test. ***P < 0.0001; ns, not significant.

IB alleles. T_1 line 1-1 and T_1 line 1-2 displayed homozygous 1-bp deletions (-C) at the *Bra j IA* sg1 site and a biallelic 1-bp insertion (+T/+A) at the *Bra j IB* sg1 target site (Table S3). It is possible that the T_0 plant was mosaic and the deleted allele was not present in the germline, whereas the detected new mutations occurred either in the T_0 or the T_1 generation. Nevertheless, these results show that the majority of our CRISPR/Cas9-induced mutations in mustard were heritable. We analyzed whether off-target mutations occurred during our editing approach. For this, 24 predicted off-target sites in the two edited T_1 lines 22-1 and 31-5 were PCR-amplified and analyzed by sequencing. No off-target mutation was detected (Figure S8).

Phenotyping of transgenic mustard seeds

The T_0 plants of both lines that clearly showed homozygous mutations (either monoallelic or biallelic) and the wild-type plants were analyzed for yield and viability traits (Table 2 and Table S2). Seed production was significantly reduced in some of the transgenic lines. This was especially obvious in the controlled crosses for which lower percentages of silique formation and much lower numbers of seeds per silique were recorded for most transgenic lines (Table S4). After open pollination, the wild-type plants of both lines produced approximately 4500 seeds per plant with a 100-seed weight of 0.257 g for Terratop and 0.289 g for CR2664, while only two out of 57

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Table 3 Genetic characterization of T_1 plants of the *B. juncea* lines Terratop and CR2664 regarding transgene presence and mutations in the target gene *Bra j I*, seed weight, and germination of seeds

Plant number	Plasmid	Line	Transgenicity assay[a]	Genotype[b] *Bra j I A* allele 1/allele 2	Genotype[b] *Bra j I B* allele 1/allele 2	Seed number obtained from free pollination	Weight of 100-seed weight [g][c]	*In vitro* germination [%] (n = 10)	*Ex vitro* germination [%] (n = 20)
K17	Wild type	CR2664	-	Wild type/wild type	Wild type/wild type	3224	0.303	100	100
K2	Wild type	Terratop	-	Wild type/wild type	Wild type/wild type	2178	0.273	100	95
22-1	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	−1 bp (sg7)/+1bp (sg7)	182	0.222	30	65
22-2	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	+1 bp (sg7)/+1bp (sg7)	85	0.125	20	30
22-3	pBraj3477	CR2664	+	−14 bp (sg7)/ −14 bp (sg7)	−1 bp (sg7)/ −1 bp (sg7)	0	n.a.	n.a.	n.a.
22-4	pBraj3477	CR2664	+	+1 bp (sg7)/ +1 bp (sg7)	−1 bp (sg7)/+1bp (sg7)	612	0.154***	50	55
22-5	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	−1 bp (sg7)/+1bp (sg7)	118	0.208	80	75
22-6	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	+1 bp (sg7)/+1bp (sg7)	256	0.120	40	30
22-7	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	−1 bp (sg7)/−1bp (sg7)	10	0.211	20	n.a.
22-8	pBraj3477	CR2664	+	−14 bp (sg7)/ −14 bp (sg7)	−1 bp (sg7)/+1bp (sg7)	521	0.181***	40	70
28-1	pBraj1256	CR2664	+	+1 bp (sg1)/ +1 bp (sg1)	−58 bp (sg1)/ −58 bp (sg1)	1199	0.381[ns]	100	100
31-2	pBraj1256	Terratop	-	−1 bp (sg1)/ −1 bp (sg1)	−4 bp (sg1)/ −1 bp (sg1)	1421	0.216***	100	95
31-5	pBraj1256	Terratop	+	−1 bp (sg1)/ −1 bp (sg1)	−1 bp (sg1)/ −1 bp (sg1)	3298	0.230***	80	90
32-1	pBraj3477	CR2664	+	+1 bp (sg4); +1 bp (sg7)/ +1 bp (sg4); +1 bp (sg7)	−2 bp (sg7)/ −2 bp (sg7)	1067	0.219***	40	40
32-2	pBraj3477	CR2664	+	+1 bp (sg4); +1 bp (sg7)/ +1 bp (sg4); +1 bp (sg7)	−2 bp (sg7)/ −2 bp (sg7)	700	0.430***	60	45
32-3	pBraj3477	CR2664	+	+1 bp (sg4); +1 bp (sg7)/ +1 bp (sg4); +1 bp (sg7)	+1 (sg7); +1 (sg4)/−2 bp (sg7)	1201	0.204***	70	80
35-3	pBraj1256	CR2664	+	+1 (sg1), −1 (sg 5)/+1 (sg1), −1 (sg 5)	−83 bp (sg5), −32 bp (sg2)/ −83 bp (sg5), −32 bp (sg2)	1037	0.156***	80	85
35-10	pBraj1256	CR2664	-	+1 (sg1)/+1 (sg1)	−83 bp (sg5), −32 bp (sg2)/ −83 bp (sg5), −32 bp (sg2)	47	0.095	n.a.	n.a.

[a]PCR with three T-DNA-specific primer pairs. +, fragment detected; -, no fragment detected.
[b]The sequencing chromatograms were decoded by ICE Analysis (https://ice.synthego.com/#/).
[c]For lines with less than 300 seeds no statistical analysis was conducted. For the remaining lines the 100-seed weight of three to four subsamples was compared against the wild type by Dunnett's test. ***P < 0.0001; ns, not significant; n.a., not analyzed.

transgenic lines produced more than 1000 seeds, i.e., lines 31 and 33 with 1264 seeds and a 100-seed weight of 0.183 g and 1303 seeds and a 100-seed weight of 0.198 g, respectively. Most of the remaining transgenic lines had a reduced seed production of less than 500 seeds per plant. The transgenic line 23 showed the lowest seed production

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with only 65 seeds and a 100-seed weight of 0.218 g. A significantly reduced 100-seed weight was observed for four T_0 plants (25, 26, 31, and 33), and three further T_0 plants produced seeds of severely reduced weight (22, 27, and 36) but due to their low seed number could not be included in the statistical comparison (Table 2 and Table S2). In contrast, seeds harvested from plant 28 were significantly heavier than those of the wild type (Table S2). The seeds from the T_0 plants were germinated *in vitro* and *ex vitro* to evaluate seed viability. *In vitro*, the T_0 lines 35 and 37 showed the highest germination rates of 100% and 80%, respectively, whereas the seeds of T_0 lines 29, 32, and 33 did not germinate at all. *Ex vitro*, the highest germination rate of 90% was observed with line 30, followed by lines 23 and 31, with a germination rate of 85%. No germination was recorded for seeds of lines 24, 29, and 34.

The seeds from those T_1 lines that carried CRISPR-induced homozygous mutations were harvested and the 100-seed weight was as well determined (Table 3). Seed production was again much lower in most T_1 lines compared to wild-type plants, and the seed weight was reduced and strongly varied from plant to plant. Seed production of the T_1 plants of line 22 ranged from 0 to 612 seeds with a 100-seed weight of 0.120 g to 0.208 g. However, the T_1 plant 31-5 of line 31 showed a production of 3298 seeds, which was even higher than that from the wild type. When testing the germination ability, the *in vitro* germination rate of the seeds of the T_1 plants of line 22 ranged from 20 to 80%, and *ex vitro* germination was comparable (from 30 to 75%). For all remaining T_1 plants, the *in vitro* germination rate varied from 30 to 100% and the *ex vitro* germination rate varied from 40 to 100%, with most T_1 lines germinating at lower percentages than wild type.

In some transgenic lines, the seed phenotype clearly differed from that of wild-type plants (Figure 3). Some T_0 and

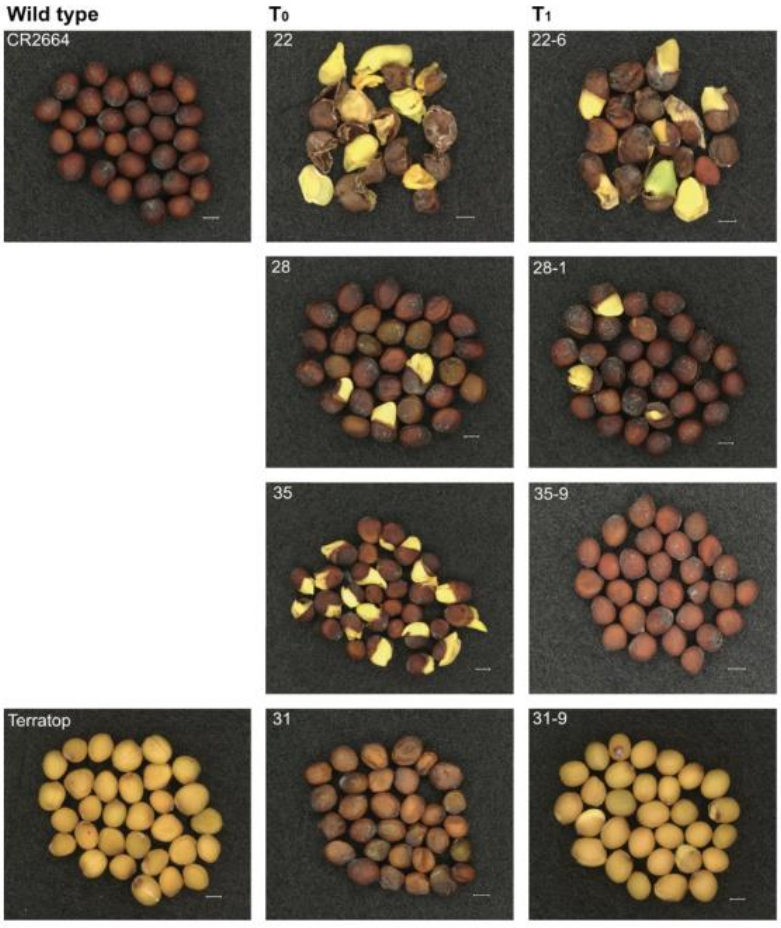

Figure 3. Seeds from wild-type, T_0, and T_1 lines of *B. juncea* (line CR2664 and Terratop) carrying the CRISPR/Cas9 constructs. Numbers 22, 28, 35, and 31 refer to T_0 lines (see Table 2 and Table S2); 22-6, 28-1, 35-9, and 31-9 refer to T_1 lines (see Table 3 and Table S3). Bar = 500 μm.

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T_1 seeds showed a precocious development of the embryo leading to a rupture of the testa and a breakthrough of the embryo through the seed coat already in the siliques. T_0 and T_1 plants of line 22 were heavily affected with more than 70% of the seeds showing this abnormality. The T_0 and T_1 plants of line 28 only rarely expressed this phenotype, whereas in line 35 the T_0 was heavily affected while the seeds of the T_1 plant lines were less affected. For the T_0 line 31 of cultivar Terratop, a change in the seed coat color was observed from yellow to brown, which reverted to yellow in some of the T_1 lines (line 31-9) (Figure 3).

Immunodetection of Bra j I in T_1 mustard seeds

The seed storage protein Bra j I is a 2S albumin with a molecular mass of about 22 kDa which is processed into two subunits of 9.5 kDa and 12 kDa (Breiteneder and Ebner, 2000; L'Hocine et al., 2019). Two bands of corresponding sizes are visible in Coomassie-stained protein profiles of seed extracts from wild-type and EGFP-transgenic plants (Figure 4a–d). These protein bands are not visible in seed extracts from T_1 lines with frameshift mutations in all *bra j I* alleles (different T1-22 lines; Figure 4a), indicating that the induced mutations successfully block Bra j I protein production. In contrast, the Bra j I protein bands are reduced in their abundance but not completely absent in the T_1 lines T1-32-1 and T1-35-3 (Figure 4b,d). Line T1-32-1 carries frameshift mutations in both *Bra j IA* and *Bra j IB*, and line T1-35-3 carries mutations in the 5′- and 3′-UTRs of *Bra j I*, but not within the coding region (Table 3).

To analyze whether the identified protein bands correspond to the Bra j I protein, two specific antibodies (KRO58-A3 and STE2-G2) were generated by antibody phage display and used in immunoblotting with seed extracts (Figure 4e–h). Both antibodies were selected against the linear immunogenic epitope of Bra j I (Figure S6) (Monsalve et al., 1993). The antibodies were cloned into the scFv-Fc format (with the human IgG1 Fc fragment) and produced in EXPI293F cells. The binding of both antibodies to complete Bra j I was validated by ELISA on recombinant Bra j I. To determine the EC_{50} values, a titration ELISA was performed (Figure S7), resulting in subnanomolar EC_{50} values for both antibodies: 0.22 nM for KRO58-A3 and 0.32 nM for STE2-G2 (KRO58-A3 was chosen for the immunoblotting). As expected, the anti-Bra j I antibody efficiently detected a protein in wild-type and EGFP-transgenic seed extracts corresponding to the heavy chain (12 kDa) of Bra j I (Figure 4e–h). No anti-Bra j I antibody binding was detected in lines T1-22 and T1-32-1, verifying that the Bra j I protein was absent in seeds of these T_1 *bra j I* mutants (Figure 4e,f).

To further estimate whether mutation of *Bra j IA*, but not *Bra j IB* results in reduced Bra j I protein accumulation within the seeds, we selected four T_1 *bra j I* mutants from T_0 line 23: T1-23-8, T1-23-12, T1-23-15, and T1-23-16, with biallelic or homozygous mutations at *Bra j IA* but no editing at *Bra j IB* (Table S3). Surprisingly, practically no Bra j I protein accumulated in these four T1-23 lines (Figure 4c,g), suggesting that the intact *Bra j IB* alleles in these T_1 lines did not result in a partial protein accumulation.

In contrast, the T_1 lines T1-31-5 and T1-35-3 showed a reduced accumulation of Bra j I protein. Lines 31-5 and 35-3 contain mutations at the 5′- and/or 3′-UTR regions of *Bra j IA* and *Bra j IB*, but not the coding regions (Table 3). This suggests that mutations in these regions might also have an impact on the level of Bra j I production. Taken together, we successfully demonstrated that several of our edited mustard lines have no or a reduced accumulation of Bra j I protein.

DISCUSSION

Transformation efficiency differs between genotypes

The genetic transformation of both mustard lines (Terratop and CR2664) using CRISPR/Cas9 editing technology was successfully achieved. In this study, we focused on the two mustard lines CR2664, originating from India with a brown testa, and Terratop, derived from a German breeder and selected for being used as a catch crop with a yellow testa. The Indian *B. juncea* lines contain higher levels of erucic acid and butenyl and propenyl glucosinolates, while the European lines were selected for a low erucic acid level and only contain propenyl glucosinolates (Lionneton et al., 2004; Vaughan et al., 1963). Fazekas et al. (1986) reported good amenability to tissue culture of the Indian lines, with greater potential for adventitious shoot regeneration than the European lines. Since the regeneration capacity is the precondition for *A. tumefaciens*-mediated transformation, most of the transformation experiments in *B. juncea* reported so far involved Indian lines (Ahmed et al., 2017; Bhuiyan et al., 2011; Dutta et al., 2008; Mondal et al., 2007; Rani et al., 2017; Sharma et al., 2004; Singh et al., 2009; Thakur et al., 2020). This paper is one of the first reporting successful *A. tumefaciens*-mediated transformation of a European line of *B. juncea*. The transformation efficiencies obtained in this study from both lines (Terratop and CR2664) varied between 0.7 and 3.8%, thus being similar to the efficiency of 0.5 to 1.5% reported by Mondal et al. (2007) but lower than the previously reported transformation efficiencies of 6–7 (Sharma et al., 2004), 16.2 (Singh et al., 2009), or 16.4 to 19.7% (Rani et al., 2017). Factors that could improve the transformation efficiencies in future experiments could involve a delayed start of selection for 3–5 days (as shown by Bhuiyan et al., 2011) or a preculture of 2 days as suggested by Thakur et al. (2020).

Successful genome editing in polyploid species

Polyploidization in flowering plants has promoted genetic variation by genome duplication during their adaptive

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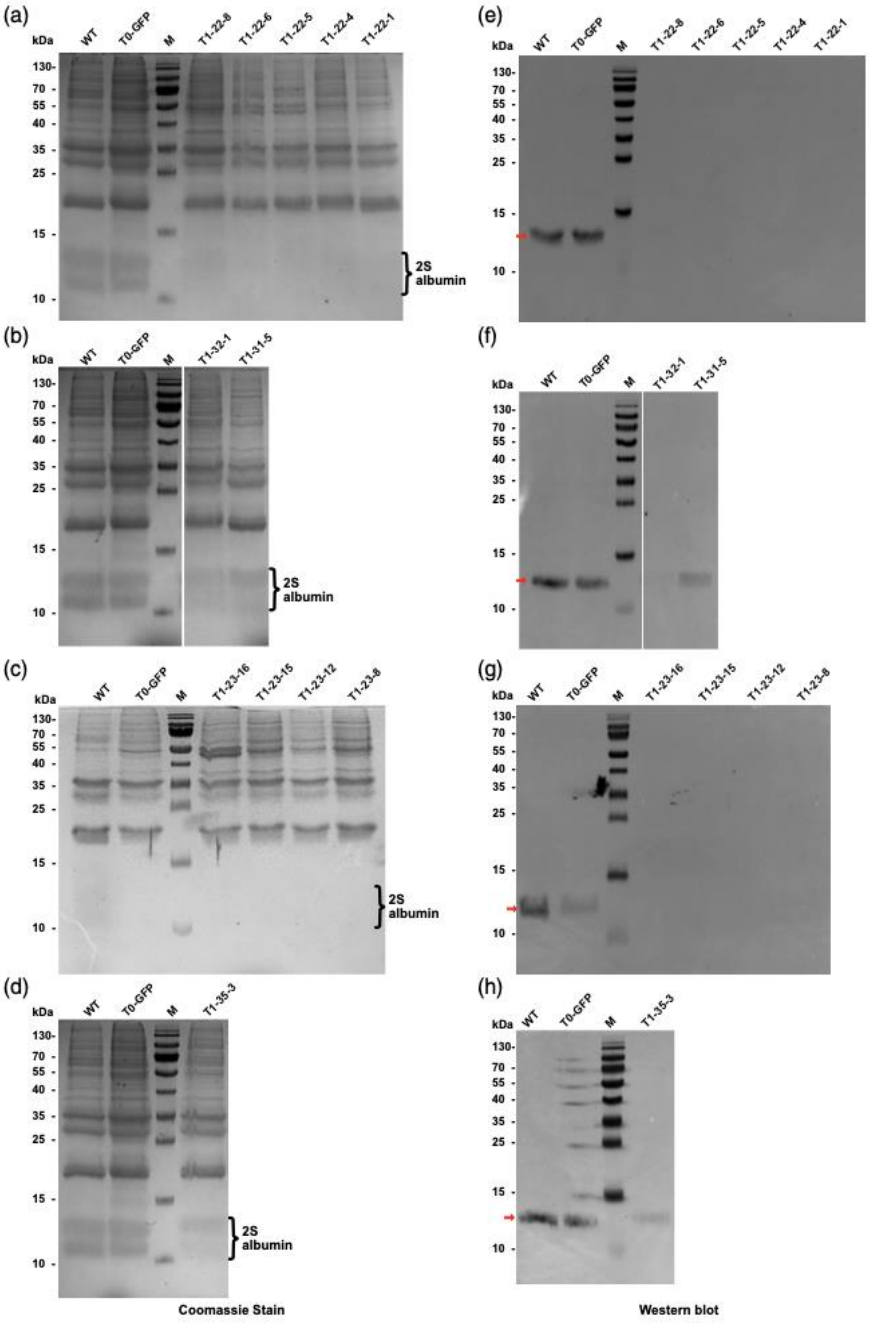

Figure 4. Loss of Bra j I protein in genome-edited mustard seeds. (a–d) Electrophoretic profiles of seed proteins from T_1 plants. The concentrations of protein extracts were normalized and about 40 μg was loaded per lane. The proteins were separated by SDS-PAGE and stained with Coomassie Blue R-250. (e–h) Immunoblot analysis of Bra j I protein in seeds from T_1 mustard plants. Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane. The Bra j I -specific monoclonal antibody KRO58-A3 was used for Bra j I (red arrow, heavy chain, approximately 12 kDa) detection. WT, wild-type CR2664 seeds; T0-GFP, EGFP-transgenic seeds (non-edited control); M, protein marker.

evolution. A large number of crops are alloploids (e.g., rapeseed [*B. napus*], wheat, and cotton [*Gossypium hirsutum*]) combining genomes from different species (Pelé et al., 2018). Random mutagenesis technologies to improve traits in polyploid crops are usually inefficient; however, targeted genome editing has become a promising

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alternative. The CRISPR/Cas9 system is the most widely used genome editing technology for precise plant genome modifications (Chen et al., 2019). Until now, CRISPR/Cas9-mediated genome editing has been successfully reported in some polyploid crops: In allotetraploid crops, the editing efficiencies varied; for example, the mutagenesis efficiency ranged from 96.8 to 100% in oilseed rape (*B. napus*) (Li et al., 2018) and from 66.7 to 100% in cotton (*G. hirsutum*) (Wang et al., 2017). A previous report showed that the Arabidopsis U6 small nuclear RNA (snRNA) promoter *AtU6-26* has a higher transcriptional activity than other *AtU6* snRNA promoters (Li et al., 2007), and in allotetraploid sweet basil (*Ocimum basilicum*) the mutation efficiency was 92.6% by using such an *AtU6-26*-sgRNA expression cassette (Navet and Tian, 2020). In this study, we utilized a multiplex CRISPR/Cas9 system (harboring the *AtU6-26*-sgRNA expression cassettes) to simultaneously edit four alleles of *Bra j I* in mustard, and the mutation efficiencies for *Bra j IA* and *Bra j IB* were 47.1 (8/17) and 50% (9/18) in Terratop and 81.3 (13/16) and 87.5% (14/16) in CR2664. These data implied that CRISPR/Cas9 is a suitable approach and our results confirmed that high editing efficiencies can be obtained also in mustard using a *35S* and *AtU6-26* promoter for *SpCas9* and sgRNA expression, respectively.

Off-target mutations are a concern for the applied use of CRISPR/Cas9. Such off-target events can be limited by using one of the high-fidelity SpCas9 variants instead of the wild-type SpCas9 (Zhang et al., 2017) or by choosing sgRNAs with few predicted off-targets (Liu et al., 2017). In plant research, possible off-target events can sometimes be removed by crossing or segregation in the next generation while keeping the desired mutations. In this study, we analyzed 24 predicted off-target sites in two edited T_1 lines and did not find any off-target mutation. Because these lines still carry the CRISPR/Cas9 transgene, it is possible that mutations will occur at a later time point or at a locus that was not analyzed. Before commercial application, a more thorough analysis might be advisable.

Altered seed phenotype in some transgenic lines

We noted that the seeds of several *bra j I* mutants were aberrant in shape (Figure 3), weight, and germination efficiency (Table 2, Table 3, Table S2, and Table S3). Those phenotypes indicate that Bra j I may have an influence on seed development. The seed yield and related traits revealed wide phenotypic variation between the wild-type plants and the transgenic T_0 and T_1. In this study, the wild types of both lines produced approximately 4500 seeds per plants with a 100-seed weight of 0.257 g for Terratop and 0.289 g for CR2664 (Table 2). The number of seeds is similar to the numbers reported by Stevens (1932, 1957) and the weight is within the range of 2.63 g and 1.84 g (1000-seed weight) reported by Stevens (1932, 1957), whereas the seed weight was higher than that recorded by Yoshimura et al. (2016) (1000-seed weight: 1.57 g). In contrast, Ramana and Ghildiyal (1997) reported much higher seed production of 3825 to 7075 per plant with a 1000-seed weight ranging from 4.49 to 4.64 g. In this study, a much lower seed production was observed for some of the transgenic lines, and this reduction was also found for the 100-seed weight and seed viability. The lower seed number might be caused by the isolation bags that had to be used for the transgenic plants as soon as flowers started to open. However, the significant reduction of seed weight and viability could also be the result of modified Bra j I levels in edited *B. juncea* seeds. Rolletschek et al. (2020) reported a seed weight reduction in transgenic lines when using RNAi to suppress seed storage protein synthesis in *B. napus*. Moreover, they observed an irregular shape of the cotyledons of mature transgenic seeds with a volume of void spaces significantly greater than in the wild-type seeds and the transgenic embryos being 7 to 9% smaller than the wild-type ones. For T_0 line 25, we could not detect a mutation in the *Bra j I* homoeologs, but observed a significantly reduced seed weight. Since this plant was proven to contain the editing construct, this fact could be explained by chimerism and a random selection of a leaf that did not carry mutations. A negative effect due to the integration site of the transgene could be an alternative explanation.

The allergen Bra j I belongs to the 2S albumin class, one of the most abundant seed storage protein classes in mustard (Monsalve et al., 1993). The seed storage proteins are essential for the seed germination vigor and provide an efficient scavenging system of the reactive oxygen species (ROS) actively generated during seed germination and, therefore, protect the other proteins that are essential for germination from oxidation (El-Maarouf-Bouteau et al., 2013; Muntz et al., 2001; Nguyen et al., 2015). Some of our T_0 and T_1 seeds showed a precocious development of the embryo leading to a rupture of the testa already in the siliques (Figure 3). Similar abnormalities were reported in F_1 hybrid seeds derived from a cross of transgenic *B. napus* and *B. juncea* that showed cracked seed coats (Tang et al., 2018). The phenotype is similar to that of mutants lacking a growth arrest during seed development and being deficient in dormancy resulting in vivipary. Vivipary occurs in mutants lacking genes that suppress germination and here especially adequate abscisic acid (ABA) levels are important to prevent sprouting (Bewley et al., 2012). The level of dormancy in a seed, i.e., its capacity to repress precocious germination, is mostly related to the capacity of the seed to synthesize ABA in the endosperm and in the embryo, which is an essential contributor to maintain dormancy (Lee et al., 2010). The reduction in seed storage protein levels in seed development often results in a low dormancy level of the seeds (Debeaujon et al., 2000; Nguyen et al., 2015; Sugliani et al., 2009). A possible link to ABA

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can be the levels of free amino acids, which can be assumed to be altered in the *Bra j I* mutant lines. Scuffi et al. (2014) were able to show that hydrogen sulfide acts as a component of the ABA signaling pathway that is involved in stomatal closure. Hydrogen sulfide can be enzymatically produced from cysteine via L-cysteine desulfhydrase (Scuffi et al., 2014).

Mutations led to the intended decreased Bra j I content in seeds

The allergen Bra j I from brown mustard was initially found in the 2S albumin fraction and recognized by the IgE of sensitive individuals (L'Hocine et al., 2019). To create Bra j I-free mustard, the *Bra j I* gene was inactivated in the genome using CRISPR/Cas9. Three regenerated T_0 *bra j I* mutant lines (lines 22, 26, and 32) were identified to contain frameshift mutations in all four *Bra j I* alleles. The corresponding seeds of T_1 offsprings of T_0 lines 22 and 32 showed significantly less 2S albumin compared to wild-type seeds. Moreover, the immunoblotting results with an anti-Bra j I antibody showed no Bra j I protein in all six of these lines, confirming that we successfully obtained Bra j I-free mustard lines. These lines are a valuable start to develop low-allergenic mustard. We noticed increased abundance of some proteins in the seed extracts of T_1 lines, e.g., T1-23-16 or T1-22-5 (Figure 4a,c), possibly indicating that the loss of Bra j I caused an accumulation of other seed storage proteins. This is in agreement with recent findings in *Camelina sativa* cruciferin C knockout lines (Lyzenga et al., 2019).

CRISPR/Cas9-induced DNA double-strand breaks (DSBs) are mainly repaired by non-homologous end joining (NHEJ) and usually result in small insertions or deletions (indels). Indels can alter the gene-coding frame and create a premature stop codon. However, indels also produce pseudo-mRNAs that do not encode functional proteins and lead to unexpected effects in CRISRP-based gene knockout studies (Tuladhar et al., 2019). Besides, such pseudo-mRNAs from the truncated reading frame of a targeted gene could produce proteins with altered amino acid sequences. Thus, a clean genetic mutation would require to completely delete a gene using two or more sgRNAs simultaneously. In our study, we expressed four sgRNAs in tandem to delete the whole *Bra j I* coding region, and Sanger sequencing confirmed that the *Bra j IB* allele was deleted by the joining of the sg1 and sg5 cleavage sites in the T_0 plant line 1 (Figure S4). However, in another line, line 47, the *Bra j IB* deletion was an outcome of an NHEJ repair with DSBs occurring in sg1 or sg2 targeted sites (Figure S4). Similar outcomes were also found in line 21 and line 56, which have large fragment deletions at cleavage sites (Figure S4). We noticed that the efficiencies of complete deletions of *Bra j IA* and *Bra j IB* were lower than we expected, and neither a deletion of *Bra j IA* nor a complete *Bra j IB* deletion line was obtained. Several studies already showed that utilizing microhomology-mediated end joining (MMEJ)-assisted CRISPR/Cas9 editing, the deletion efficiency can be increased (Owens et al., 2019; Tan et al., 2020). An alternative approach might thus be to design sgRNAs targeting microhomologous sequences to induce a complete deletion of *Bra j IA* and *Bra j IB* by the MMEJ DNA repair mechanism.

CONCLUSIONS AND OUTLOOK

The Bra j I mutation lines that were generated in this work now serve as a key starting point to further study the impact of changes in seed storage protein composition in mustard. Future work will correlate specific changes in seeds with their allergenic potential for patients and their food quality. In principle, it might even be possible to precisely change epitopes in proteins that trigger IgE binding using precision genome editing (base editors or prime editors; Anzalone et al., 2020) without removing the protein altogether and thereby avoiding altered seed phenotypes.

The protocols established for genetic transformation and CRISPR/Cas9-mediated genome editing in *B. juncea* pave the way for application in modern breeding programs of this allotetraploid crop. Compared to traditional breeding, genome editing-based new breeding technologies accelerate crop improvement since they can generate precise genetic changes and desired traits in complex genetic backgrounds in a relatively short time (Gao, 2021). Our work demonstrates the removal of an allergenic protein from brown mustard potentially improving the safety of mustard-derived food products for sensitized individuals. This work also highlights the possibility for creating hypoallergenic food plants in general to enhance their safety for human consumption.

EXPERIMENTAL PROCEDURES

Plant material and explant preparation

Seeds of two brown mustard (*B. juncea* L.) lines were used, the European cultivar Terratop (kindly provided by the breeder P.H. Petersen Saatzucht Lundsgaard GmbH, Grundhof, Germany) and an Indian line of *B. juncea* (L.) Czern. with accession number CR2664 obtained from the gene bank of the IPK Gatersleben, Germany. The mature seeds of both lines were surface-disinfected with 1% NaOCl with 0.1% Tween 20 for 5 min followed by three washing steps with sterile deionized water under aseptic conditions. The treated seeds were germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) for 5 days in darkness at 24°C. The 5-day-old seedlings were collected under aseptic conditions, and the cotyledons were vertically divided into two halves and used as explants for the genetic transformation experiments.

Vector constructs

All the vectors used in this work were assembled based on Modular Cloning-compatible vectors (Table S5) (Weber et al., 2011). In

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short, to construct pEGFP, 2x*35S*::*nptII* and 2x*35S*::*EGFP* transcriptional units were ligated at positions 1 and 2, respectively, into pAGM8031 by Golden Gate assembly. Suitable target sequences of sgRNAs (Table S6) were chosen using the online web tool CRISPR-P (Liu et al., 2017). Complementary oligos with target sequences were synthesized by Microsynth AG (Balgach, Switzerland). Oligonucleotide pairs were annealed and cloned into an intermediate vector (pDI1, pDI2, pDI3, and pDI4E) with *Bpi*I, yielding *AtU6-26*::*sgRNA* expression cassettes. The 2x*35S*::*HPT* transcriptional unit and the 2x*35S*::*SpCas9* transcriptional unit together with four sgRNA (sg1, sg2, sg5, sg6) expression cassettes were ligated into pAGM8031 by Golden Gate assembly, yielding the final construct pBraj1256. The 2x*35S*::*HPT* transcriptional unit and the 2x*35S*::*SpCas9* transcriptional unit and four sgRNA (sg3, sg4, sg7A, sg7B) expression cassettes were ligated into pAGM8031 by Golden Gate assembly, resulting in the final construct pBraj3477. The final constructs were transformed into *A. tumefaciens* strain LBA4404 for transformation of plants. All oligonucleotides used in this work are listed in Table S7.

Transformation of mustard plants

Glycerol stocks of the *A. tumefaciens* strain LBA4404 harboring the binary vectors pEGFP, pBrj1256, and pBrj3477 were plated on YEB medium (0.5% [w/v] sucrose, 0.5% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] beef extract, 0.05% [w/v] magnesium sulfate, 1.4% [w/v] micro agar, pH 7.2) supplemented with 100 mg L^{-1} rifampicin and 100 mg L^{-1} spectinomycin and incubated for 24 h at 28°C. The bacteria were then collected from the plates and dissolved in simplified induction medium (Alt-Moerbe et al., 1988) containing 20 mM sodium citrate and 2% sucrose (pH 5.2) supplemented with 100 μM acetosyringone and 1 mM betaine hydrochloride (James et al., 1993) and incubated at room temperature for 4 h until an OD_{600} of 1.8 to 2.0 in the 1:10 dilution was reached. Medium without bacteria was used for control variants. The cotyledon explants were immersed in the different solutions supplemented with 0.03% Silwet L-77 for 10 min, blotted dry on sterile paper towel, and co-cultivated on shoot induction medium (full-strength MS medium supplemented with 3% [w/v] sucrose, 0.8% [w/v] agar, 8.88 μM benzyladenine, 5.37 μM naphthalene acetic acid, and 100 μM acetosyringone) for 3 days. After co-cultivation, the explants were washed in sterile deionized water and placed onto regeneration medium (shoot induction medium + 10 μM $AgNO_3$) and the different selection media depending on the construct (shoot induction medium + 10 μM $AgNO_3$ + 15 mg L^{-1} hygromycin for Terratop or 20 mg L^{-1} hygromycin for CR2664 with the CRISPR/Cas9 constructs and 50 mg L^{-1} kanamycin for both lines with the EGFP construct) for shoot organogenesis at 24 ± 1°C with a 16/8 h day/night cycle (35–40 μmol m^{-2} sec^{-1}, fluorescent tubes). After 28 days, the obtained shoots were separated and cultivated on the same media without $AgNO_3$ for 28 days. Surviving shoots were subcultured every 4 weeks for another 56 days. Four months after co-culture, putative transformants (T_0) were obtained. The plants from the non-transformed control (wild type) and the T_0 transgenic plants were acclimatized and grown in 5-L pots containing growing medium (3:1 mixture of peat and lime-free clay and fertilized with 1 ‰ Ferty3 Mega produced by Einheitserdewerke Patzer, Patzer Erden GmbH, Germany) under a 16/8 h day/night cycle using a Philips SON-T Agro 400 W at 22°C in the greenhouse for 4 months. Before anthesis of the first flowers, the plants were covered with isolation bags (Crispac-Beutel SM 330 × 750 mm, Baumann Saatzuchtbedarf GmbH, Germany) and shaken from time to time to improve self-pollination. Where possible, 20 flowers per plants were emasculated before anthesis and the anthers with mature pollen grains were collected from other flowers of the same plant for pollination (Table S4). At maturity, seeds were harvested to obtain the seeds for the T_1 generation.

Seed germination

The germination rates of seeds from wild-type, T_0, and T_1 transgenic plants were determined *in vitro* and in the greenhouse. For *in vitro* germination, 10 seeds from each plant were randomly chosen, subjected to surface disinfection, and incubated in darkness for 7 days at 24°C. A seed was scored as germinated 7 days after sowing when the radicle and the hypocotyl had emerged. For *ex vitro* germination, 20 seeds from wild-type, T_0, and T_1 transgenic plants were directly sown into multipot trays or 8-cm pots containing growing medium (3:1 mixture of peat and lime-free clay and fertilized with 1 ‰ Ferty3 Mega produced by Einheitserdewerke Patzer, Patzer Erden GmbH, Germany) under a 16/8 h day-night cycle using a Philips SON-T Agro 400 W, at 22°C in the greenhouse. The germination was scored after 21 days when the cotyledons had emerged above the substrate.

Seed characterization

The harvested seeds from each plant were counted with a counting machine (Contador, Pfeuffer, Kitzingen, Germany) to quantify the seed set per plant. Where possible, four times 100 seeds from each individual plant were randomly selected and the weight was determined using a precision balance (Denver Instrument SI-203.1, Cole-Parmer GmbH, Germany). Later, 15 to 30 seeds were randomly selected and photographed with a digital microscope (Figure 3) (VHX-S750E, Keyence, Japan).

Statistical analysis was conducted on the 100-seed weight using R (R Core Team, 2021) with the help of the packages tidyverse (Wickham et al., 2019) and dplyr (Hadley et al., 2021). The R package emmeans was also used to conduct pairwise comparisons (Dunnett test) (Lenth, 2021).

Identification of mutants

Frozen leaf samples were disrupted with a TissueLyser (QIAGEN GmbH, Germany) and genomic DNA was extracted using the innuPREP Plant DNA Kit (Analytik Jena, Germany) according to the instructions of the manufacturer. Three pairs of primers (hyg-F/hyg-R, F1/R1, and F2/R2) were used to detect transgenic mustards with T-DNA integrated into the genome. Subsequently, *Bra j IA* and *Bra j IB* were amplified by Q5 High-Fidelity Polymerase (NEB) from genomic DNA with site-specific primer pairs BrajA-F/BrajA-R and BrajB-F/BrajB-R (Table S7), respectively. The PCR amplicons were purified from the agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific Fermentas, USA) and sequenced (Microsynth Seqlab, Göttingen, Germany) or subcloned into pUC57 vector before sequencing. The Sanger sequencing chromatograms were decoded manually and using the ICE Analysis tool from SYNTHEGO (https://ice.synthego.com).

Generation of a monoclonal Bra j I-specific antibody

Antibody selection was performed as described previously (Russo et al., 2018) with modifications. In brief, antibodies were selected by phage display using the naïve antibody gene libraries HAL9 and HAL10 (Kügler et al., 2015) on a biotinylated peptide (Monsalve et al., 1993) (Peps4LS GmbH, Heidelberg, Germany) in microtiter plates (Corning, New York, USA). Selected antibodies were cloned into scFv-Fc format and were produced in EXPI293F cells as previously described (Wenzel et al., 2020). Titration ELISA

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was performed with the selected antibodies. For a more detailed description see Supplementary Material.

Immunoblotting

Seeds were ground into a fine powder with 25% (w/v) extraction buffer (0.2 M Tris-Cl, pH 8.0, containing 0.1 M NaCl, 10 mM EDTA, 0.1% $MgCl_2$, 10% glycerol, 1 mM PMSF, and 10 nM β-mercaptoethanol) and centrifuged at 14 000 ***g*** for 10 min at 4°C. The supernatants were transferred to new tubes and centrifuged again at 11 304 ***g*** for 10 min at 4°C. These supernatants were used for further analysis. Total protein concentrations were measured using Bradford reagent (Carl Roth GmbH, Karlsruhe, Germany). Samples were boiled with loading dye at 95°C for 10 min. Protein samples were separated by 15% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250. For Bra j I detection, proteins were transferred onto 0.45 μm nitrocellulose membranes using a wet blotting system. Membranes were blocked overnight at 4°C in blocking buffer (TBST buffer with 5% defatted milk and 3% bovine serum albumin). Subsequently, membranes were incubated with 100 ng ml^{-1} primary antibody for 3 to 4 h at room temperature. After washing the membranes three times for 5 min each in TBST buffer, they were incubated with 1:20 000 diluted alkaline phosphatase-conjugated goat anti-human IgG secondary antibody (Dianova 109-055-98) for 1 h at room temperature. Then, the membranes were washed three times with TBST buffer for 5 min each and equilibrated with substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 10 min. Thereafter, the membranes were stained with NBT/BCIP substrate staining solution and the staining reaction was stopped by washing three times with H_2O after the signal developed. Images were taken by a ChemiDoc™ Touch Imaging System (Bio-Rad).

Off-target detection

Off-target sites were predicted by the online tool CRISPR-P (Liu et al., 2017). Based on the off-target score, the top three predicted off-target sites for each of the eight used sgRNAs were selected as potential off-target sites (Figure S8). Those sites were amplified from the genome of two selected T_1 plants by PCR and the PCR product was sequenced. Primer pairs for off-target amplification are added to Table S7.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

TW, JB, and MH conceived the study and its design, coordinated the research together with TR, and assisted with interpretations of results. JA, DZ, KR, and SS designed and performed the experiments and analyzed the data. JA, DZ, TW, JB, and KR wrote the manuscript. TR provided the recombinant Bra j I protein.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the article and supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Sequence alignment of *Bra j I* homoeologs in *B. juncea* varieties Terratop and CR2664.

Figure S2. Outcomes of the PCR test for transgenicity of all obtained mustard lines.

Figure S3. PCR amplification of both *Bra j I* homoeologs from genomic DNA of the T_0 lines.

Figure S4. Analysis of deletions in *Bra j I* in mutated T_0 lines.

Figure S5. Analysis of the *Bra j I* homoeologs of T_1 offsprings to screen for inheritance of the deletion from the T0-1 line.

Figure S6. Amino acid sequence alignment of Bra j I homoeologs in *B. juncea* varieties Terratop and CR2664.

Figure S7. Titration-ELISA for EC_{50} determination on Bra j I peptide.

Figure S8. Off-target detection.

Table S1. Transformation efficiencies for two *B. juncea* lines (Terratop and CR2664) in different experiments using the three vectors pEGFP, pBraj1256, and pBraj3477.

Table S2. Genetic characterization of T_0 plants regarding transgene presence, seed weight, and germination of seeds of the *B. juncea* lines Terratop and CR2664.

Table S3. Genetic characterization of T_1 plants regarding transgene presence, seed weight, and germination of seeds of the *B. juncea* lines Terratop and CR2664.

Table S4. Controlled pollinated flowers of T_0 plants of *B. juncea* lines Terratop and CR2664 with the number of seeds and seed weight.

Table S5. MoClo-compatible vectors used in this study.

Table S6. sgRNA sequences.

Table S7. Oligonucleotides used in this study.

Supplementary Material. Antibody selection in microtiter plate.

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4 Effect of cytokinins and light quality on adventitious shoot regeneration from leaflet explants of peanut (*Arachis hypogaea*)

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Hans Bethge	Designed and implemented materials, contributed to the writing of the manuscript
David Wamhoff	Contributed to the analysis tools, contributed to the writing of the manuscript
Traud Winkelmann	Conceived and designed the experiments, contributed to the writing of the manuscript

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ABSTRACT

Plant tissue culture and novel breeding techniques such as genome editing in the economically important crop *Arachis hypogaea* constitute potential for genetic improvement. Therefore, in this study, high-frequency *in vitro* regeneration via formation of adventitious shoots was described for different *A. hypogaea* lines. The leaflets of the primary leaves of 5-day-old seedlings were cultured for 16 weeks on Murashige and Skoog's medium supplemented with equimolar concentrations (22.19 µM) of the cytokinins 6-benzylaminopurine (BAP); thidiazuron (TDZ); zeatin (Zea) or meta-topolin (MT), which was reduced to 7.40 µM after eight weeks plus 2.3 µM kinetin. Highest shoot regeneration percentages of 89.1% and 81.3% for BAP and TDZ containing media, respectively, were achieved in the line 'Jimmy's pride' with the maximum shoot number obtained on BAP. The regeneration percentages for three further lines were lower than 50%. Furthermore, the effect of different light qualities, applied using LEDs, and compared to fluorescent lamps was investigated. Shoot regeneration percentages did not improve significantly under the different LEDs, instead a pronounced effect on the regeneration efficiency of the culture temperature was observed which differed among the light treatments. However, fluorescent lamps can be replaced by red or blue LEDs without negative effects on shoot regeneration.

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In vitro culture; light-emitting diodes; organogenesis; plant growth regulators; temperature; tubular fluorescent lamps

Introduction

The genus *Arachis*, belongs to the angiosperm family Fabaceae and contains about 80 described species (Krapovickas & Gregory, 2007; Yol et al., 2018). In contrast to other flowering plant genera, the genus *Arachis* is geocarpic: the fruits are produced below ground, whereas flowers and stems are formed above ground (Krapovickas & Gregory, 2007). The cultivated peanut (*Arachis hypogaea*) is an annual amphidiploid (AABB, 2n = 40) crop, with *Arachis duranensis* and *Arachis ipaensis* as the diploid donor species of the AA and BB sub-genomes, respectively (Bertioli et al., 2016). Peanut originated from South America, from the region of eastern foothills and the Andes (from southern Bolivia to northwestern Argentina) (Banks, 1976; Variath & Janila, 2017). Nowadays it is cultivated worldwide, in central America and the Caribbean region as well as in Asia and Africa (Caballero et al., 2003).

Peanut is the second most significant cultivated grain legume, and the fourth largest oilseed crop in the world (Shilman et al., 2011). Peanut is produced worldwide for oil, nuts, peanut butter and candy. *Arachis hypogaea* (L.) is separated into two subspecies, *hypogaea* and *fastigiata* (Krapovickas & Gregory, 2007). Four types of peanuts with distinctive size, oil content and flavour are mostly cultivated worldwide, the types Virginia and Runner belonging to the subsp. *hypogaea* and the Spanish and Valencia types of the subsp. *fastigiata* (Yol et al., 2018).

The cultivated peanut is an excellent reservoir of seed oil (~40–50% of dry seeds), with high-quality protein (~20–35%) and carbohydrates (~10–20%) (Janila et al., 2016; Variath & Janila, 2017). Interest is given in peanut lines with high oleic acid content which are less prone to oxidation at ambient storage temperatures and also at the high temperatures, which prevail during roasting, cooking, and frying and thus, are more resistant to discoloration (Shilman et al., 2011; Uematsu et al., 2002; Zainuddin et al., 2004). Peanut is an important source of fat-soluble vitamins A, D, E, K and water-soluble vitamins (B-complex and C), other fundamental minerals (Ca, P, Mg, Zn and Fe) (Kassa et al., 2009; Settaluri et al., 2012; Variath & Janila, 2017) and phenolic compounds (Francisco & Resurreccion, 2008; Variath & Janila, 2017). Peanut oil is as well used in the industry for various purposes namely, cosmetics, lubricants, furniture polish, insecticides, nitroglycerine, soap, varnish and leather dressings (Variath & Janila, 2017).

However, two main limitations, namely, aflatoxin contamination and allergens are detrimental when it comes to human consumption (Variath & Janila,

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2017). Peanut proteins are described as major sources of allergens with peanut seed consumption reported to be one of the most important causes of fatal food-induced anaphylaxis (Yocum & Khan, 1994). This affects 0.4% to 1.4% of people worldwide, especially children (Palladino & Breiteneder, 2018). Therefore, developing peanut cultivars with reduced allergenic potential is an important objective for research and breeding. Conventional peanut breeding faces many difficulties due to polyploidy and sterility barriers (Wilson et al., 2019). However, the availability of the complete peanut genome sequence (Bertioli et al., 2019) represents a big step towards developing varieties through CRISPR/Cas-based genome editing technology, targeting specific traits such as pest resistance, high nutritional value, high yield and tolerance to abiotic and biotic stressors. Neelakandan et al. (2022) reported successful targeted mutagenesis of the *FAD2* gene to increase the content of oleic acid in peanut using CRISPR-based gene editing, whereas Biswas et al. (2022), used a polyethylene glycol (PEG)-mediated protoplast transformation system as a pilot study of CRISPR-based gene editing in peanut targeting the allergen gene *Ara h 2*.

The genetic transformation of CRISPR/Cas vectors requires an efficient and reproducible regeneration system, either via organogenesis or via somatic embryogenesis. In previous studies, considerable effort has been reported to induce shoots from several plant tissues of *Arachis hypogaea*, often derived from young seedlings, namely, cotyledons (Baker & Wetzstein, 1995; Cucco & Rossi Jaume, 2000; Durham & Parrott, 1992; Masanga et al., 2017; McKently et al., 1990; Ozias-Akins et al., 1992; Pestana et al., 1999; Radhakrishnan et al., 2000; Tiwari & Tuli, 2012; Tiwari et al., 2015), cotyledonary nodes (Anuradha et al., 2006; Banerjee et al., 1988, 2007; Bhatnagar et al., 2010; Hsieh et al., 2017; Sharma & Anjaiah, 2000), embryo axes (McKently, 1995; Ozias-Akins et al., 1992; Radhakrishnan et al., 2000), immature embryos (Hazra et al., 1989; Ozias-Akins, 1989), leaflets (Baker & Wetzstein, 1992, 1998; Cucco & Rossi Jaume, 2000; Joshi et al., 2003; Tiwari & Tuli, 2009; Venkatachalam et al., 1999), leaf discs (Akasaka et al., 2000; Eapen & George, 1993), epicotyls (Cheng et al., 1992; Cucco & Rossi Jaume, 2000; Hongwei & Jinhua, 2003; Little et al., 2000; Shan et al., 2009) and hypocotyls (Matand & Prakash, 2007; Radhakrishnan et al., 2000; Venkatachalam et al., 1997). Nevertheless, peanut is still considered as difficult-to-regenerate, as are most legume species. Moreover, for those explants containing the apical or lateral shoot meristems, the shoots formed are not necessarily of adventitious origin resulting in a high risk of chimeral regenerants in transformation approaches. The shoot formation efficiencies strongly differed among the studies and were strongly influenced by several factors such as the genotype, the plant growth regulators, the age of the seedlings, the explant type and the culture temperature.

More recently, light quality has become an interesting growth factor in plant tissue culture, being tightly linked to plant hormone signalling and controlling plant morphogenesis and cell growth (Cavallaro et al., 2022). The commonly used light source in plant *in vitro* culture are still tubular fluorescent lamps (TFLs) providing different light spectra (350–750 nm) and consume over 65% of the total electricity in tissue culture laboratories (Yeh & Chung, 2009). Strong photon densities are seen in the infrared and red areas of the TFLs, and they gradually diminish towards the blue region. Because of the phosphor coating, white TFLs also display a continuous visible spectrum with peaks at 400–450 nm (violet – blue), 540–560 nm (green – yellow), and 620–630 nm (orange-red) (Cavallaro et al., 2022). However, the primary drawbacks associated with the use of the TFLs are that, substantial portion of the spectral output that is not required for plant morphogenesis since they are abundant in green and yellow, which are less effective for plants and lack sufficient far-red light (Dutta Gupta & Jatothu, 2013; Miler et al., 2019).

Light quality affects plant developmental processes, such as shoot formation, rooting or chlorophyll and carotenoid pigment synthesis (Bello-Bello et al., 2017; Cavallaro et al., 2022; Dewir et al., 2005; Li et al., 2010, 2012). However, the effect of the light quality on the physiological and morphological aspects of plant development is species-dependent (Dewir et al., 2005) and investigations in this field just recently started. Red light (660 nm) and far-red light (730 nm) activate and inactivate reversible phytochromes and blue light (460 nm) activates cryptochrome photoreceptors, and both pigments are involved in regulating plant morphology and gene expression (Martínez-García et al., 2000). Likewise, red light was shown to have positive effects in promoting adventitious shoot bud formation in *Stevia rebaudiana* (Ramírez-Mosqueda et al., 2017) and *Lactuca sativa* (Kadkade & Seibert, 1977). Although blue light effects can counteract those of red light, positive effects of blue light were reported resulting in promotion of *in vitro* shoot induction in *Gerbera jamesonii* 'Rosalin' (Gök et al., 2016), from *Nicotiana tabacum* callus (Weis & Jaffe, 1969), and increasing the shoot number formation in *Ficus benjamina* (Werbrouck et al., 2011) and *Hyacinthus orientalis* L. (Bach & Świderski, 2000).

The objective of this study was the establishment of an efficient adventitious shoot regeneration system in *Arachis hypogaea* (L.) for later use for genetic transformation. In order to avoid axillary shoot formation, we used very young primary leaflets of seedlings of four peanut lines, the cultivar 'Jimmy's Pride', the breeding line 'Dhaka', and two gene bank accessions,

Table 1. Cytokinins tested in shoot induction media (SIM) for adventitious shoot regeneration from the primary leaf explants of *Arachis hypogaea*. BAP: 6-benzylaminopurine, MT: meta-topolin, TDZ: thidiazuron, Zea: zeatin, Kin: kinetin. Duration of all culture passages: 4 weeks.

Concentration (µM)						
Medium	Culture passage	BAP	MT	TDZ	Zea	Kin
SIM1	1 & 2	22.19				2.3
	3 & 4	7.40				2.3
SIM2	1 & 2		22.19			2.3
	3 & 4		7.40			2.3
SIM3	1 & 2			22.19		2.3
	3 & 4			7.40		2.3
SIM4	1 & 2				22.19	2.3
	3 & 4				7.40	2.3

one Valencia and one Runner type. The study focused on studying light as a factor influencing organogenesis. Therefore, in-house constructed LED modules were employed that allowed a wireless control of light intensity, light quality and photoperiod. The emitted wavelengths of the LEDs used in this study (blue at 440 nm, green at 520 nm, red at 660 nm, far red at 730 nm) were selected based on the absorption maxima of the major plant photoreceptors. Besides, the effect of different types of cytokinins on adventitious shoot regeneration from peanut leaflets was also investigated.

Materials and methods

Plant materials and culture media

Seeds of four peanut (*Arachis hypogaea* L.) lines were used for the experiments: The cultivar 'Jimmy's Pride' was kindly provided by Dr. Thomas Reinard, Institute of Plant Genetics at Leibniz University Hannover, Germany, whereas the breeding line 'Dhaka' was generously made available by Prof. Imdadul Hoque, Institute of Biological Science at the University of Dhaka, Bangladesh. The types Valencia and Florunner with the accession numbers PI336942 (abbreviated as G8) and PI565448 (G12), respectively, were obtained from the gene bank of the United States Department of Agriculture (USDA) and seed-propagated in the greenhouses of the Leibniz University Hannover.

The seeds were carefully washed in water and commercial detergent (0.5 mL/L) for 30 min, then surface-disinfected by stirring in 4.5% NaOCl with 0.1% Tween 20 for 20 min, and subsequently rinsed 3 times with sterile deionised water under aseptic conditions in a laminar air flow. Thereafter, the seeds were germinated for five days at 27°C in darkness in 150 ml glasses (one seed per vessel), covered by twist-off lids lined with filter papers, containing 25 mL germination medium. The plant growth regulator-free germination medium contained half-strength MS (Murashige & Skoog, 1962) salts and full-strength MS vitamins with 3% (w/v) sucrose and 0.2% (w/v) glucose and was solidified by 0.4% (w/v) gelrite (Duchefa, Haarlem, The Netherlands). The pH was adjusted to 5.5–5.6 before autoclaving at 120 ± 1°C for 20 min.

In the experiments aiming at adventitious shoot induction described below, full-strength MS medium and B5 vitamins (Gamborg et al., 1968), with 3% (w/v) sucrose and 0.8% (w/v) Plant agar (Duchefa, Haarlem, The Netherlands) were used. For experiment 1, four media with the cytokinins 6-benzylaminopurine (BAP), thidiazuron (TDZ), zeatin (Zea) and meta-topolin (MT: 6-(3-hydroxybenzylamino) purine), at the same concentration of 22.19 µM and reduced to 7.40 µM eight weeks after culture initiation, each supplemented with kinetin (Kin: 2.3 µM) were compared (Table 1).

Explant preparation and culture conditions

The leaflets of the primary leaves were carefully excised from the 5 days-old seedlings and used as explants (Figure 1).

For the first experiment, the explants were incubated in a climatic chamber (RUMED® from Rubarth Apparate GmbH, Germany) at a temperature of 27 ± 1°C and at a 16 h photoperiod under a photon flux density (PPFD-PAR) of 50 ± 20 µmol $m^{-2}s^{-1}$ provided by tubular fluorescent lamps (TFLs) (Philips MASTER TL-D 58W/840). Whereas for the second and third experiment, the explants were cultured in a custom lighting module consisting of six compartments each equipped with an LED lighting system positioned at the top and emitting specific light qualities. For the experiments, a 16 h photoperiod was chosen under a photon flux density ($PFD_{350\text{-}800\ nm}$) of 20 and 29 µmol $m^{-2}s^{-1}$ for experiment 2 and 3, respectively (Figure S4). Light intensities and qualities were measured with a radiometric and wavelength-calibrated spectrometer (Ocean optics USB 4000 UV-VIS) in the spectral range of 350 nm to 800 nm.

In all experiments, the explants were cultured for 16 weeks, and sub-cultured every 4 weeks. The explants were subjected to different culture conditions depending on the experiment. The first two culture passages were realised in 9 cm polystyrene Petri dishes

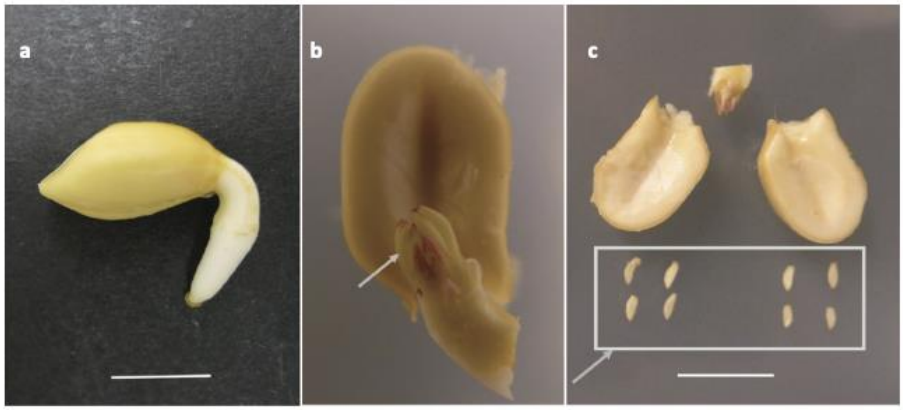

Figure 1. Explant preparation. a: 5-day-old seedling; b: radicle and one cotyledon removed; c: cotyledons, embryo axis and leaflets of the two primary leaves. Arrows point to the explants used: leaflets of primary leaves, bars indicate 1 cm.

containing 33 ml medium, whereas in culture passages 3 and 4, 250 ml polypropylene vessels containing 70 ml medium were used.

Description of the LED research module

The LED research modules (Bethge, 2018; Bethge et al., 2018) each consisted of 24 insulated metal substrate printed circuit boards (IMS-PCB), containing up to six LEDs emitting different wavelengths (UVB 305 nm, blue 445 nm, green 524 nm, red 659 nm, far red 726 nm and white with a colour temperature of 6500 K as a control variant for the fluorescent lamps) (Figure 2). The emission peak maxima had been selected based on the absorption maxima of the major plant photoreceptors. The light modules were designed to provide homogeneous light distribution within the experimental area of a shelf (coefficient of variation Ø: ~4%). Using the LED-research module, radiation intensities could be selected in the range between 0 and 200 µmol $m^{-2}s^{-1}$. Light qualities as a combination of the emission wavelengths of the LEDs in the range between 300 and 730 nm, any photoperiod and frequencies of the pulse-width-regulated (PWM) LEDs in the range from 100 to 1000 Hz could be applied to the explants.

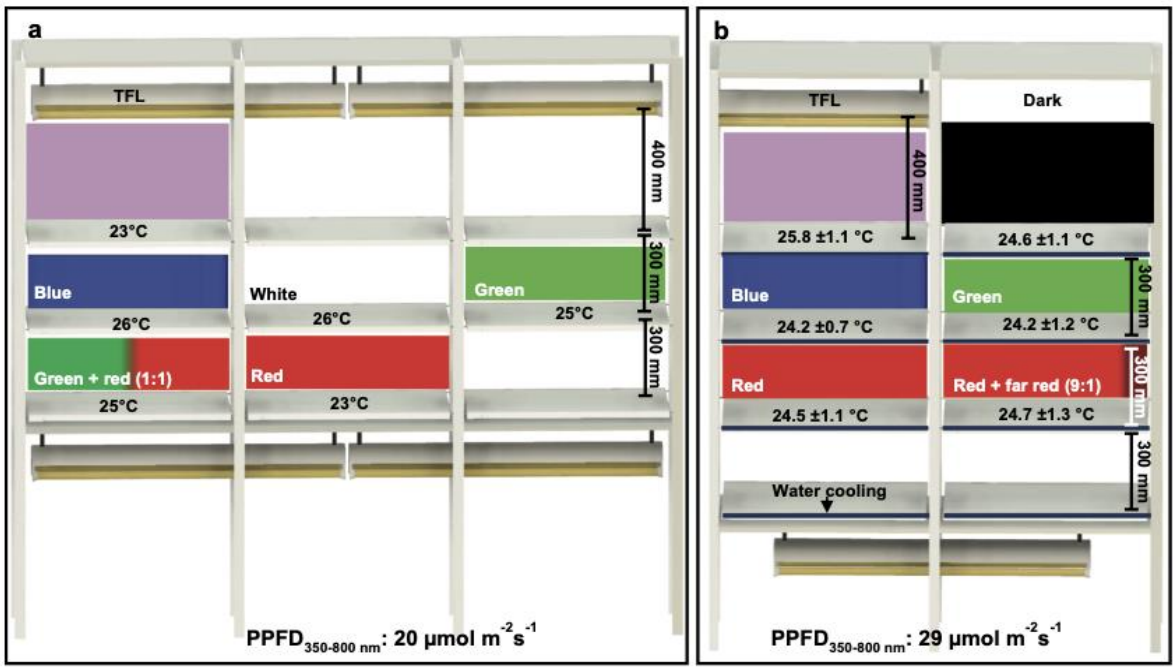

Figure 2. The light-emitting diode (LED) system and the design of the custom lighting module used in this study, a) spatial arrangement of modules in experiment 2, b) spatial arrangement of modules in experiment 3 with the use of an active water cooling of the cultivation surface to provide homogeneous temperature for all variants, TFL: tubular fluorescent lamps.

In this study, two spatial arrangements of the LEDs research modules were used: experiment 2 was conducted with arrangement A (Figure 2), and experiment 3 with arrangement B. To exclude residual light, each light variant was fully covered with black foil in both experiments. Moreover, in experiment 3, an active water-cooling system was implemented by water-carrying copper pipes, microcontrollers and solenoid valves responding to a sensor, measuring the surface temperature of the cultivation area.

Experimental set up

Experiment 1: Effect of cytokinins on adventitious shoot regeneration

The shoot regeneration potential of the four peanut lines was compared, by testing different cytokinins (BAP, TDZ, Zea and MT) at an equimolar concentration (22.19 µM, concentration based on Pestana et al., 1999), which was reduced to 7.40 µM 8 weeks after culture initiation to promote the outgrowth of shoot buds and to reduce the risk of somaclonal variation. Per variant, a total of 80 primary leaf explants were cultured in 10 Petri dishes with 8 explants each.

Experiment 2: Effect of different light qualities on adventitious shoot regeneration

The influence of the different light qualities on adventitious shoot formation was tested with three peanut lines ('Jimmy's Pride', PI336942 and PI565448). Line Dhaka could not be included in this experiment due to seedling contaminations. The light and temperature conditions are summarised in Table 2. Ten Petri dishes with 8 primary leaf explants each and the best regeneration medium from the experiment 1, SIM1 with 22.19 µM BAP +2.3 µM Kin for the first 8 weeks and 7.40 µM BAP +2.3 µM Kin thereafter (Table 1) were used per light variant and per line. The temperatures under the light variants were measured during the experiment (Table 2).

Experiment 3: Effect of different light qualities with controlled temperature on adventitious shoot regeneration

For experiment 3, adventitious shoot regeneration of the four peanut lines was investigated using the custom lighting module with the bottom cooling system to standardise the temperature in all LED compartments (Table 3). A total of 80 primary leaf explants per light variant and line (ten Petri dishes with eight explants each) and medium SIM1 (Table 1) were used for this experiment.

Evaluation of the adventitious shoot regeneration

In all experiments, the explants were cultured for 16 weeks and the adventitious shoot formation (yes/no) was evaluated every 4 weeks. At each evaluation, the explants were subcultured and contaminated explants were discarded. At the fourth evaluation, data of shoot formation were recorded and consisted of calculating (1) the frequency of adventitious shoot formation [%] per Petri dish (= replicate) by dividing the number of explants forming adventitious shoots by the total number of explants in the Petri dish and (2) the mean number of adventitious shoots formed per regenerating explant based on the number of explants forming shoots.

Statistical analyses

Statistical analyses were performed using the R program (version 4.0.5, R Core Team, 2021). Packages tidyverse (Wickham et al., 2019) and dplyr

Table 2. Overview of the light variants and the physical growth conditions in experiment 2.

Light variant	Emission peak maximum [nm]	Photon ratio	Light intensity (µmol/m²/s)	Temperature (°C)
Blue	444	1	20	26
Green	524	1	20	25
Red	659	1	20	23
White	6500 K[a]	1	20	26
Green & red	524 & 659	1 + 1	20 (10 + 10)	25
Tubular fluorescent lamp	6500 K[a]	1	20	23

[a]Colour temperature in Kelvin.

Table 3. Overview of the light variants and the physical growth conditions in experiment 3.

Light variant	Emission peak maximum [nm]	Photon ratio	Light intensity (µmol/m²/s)	Temperature (°C)[b]
Blue	444	1	29	24.2 ± 0.7
Green	524	1	29	24.2 ± 1.2
Red	659	1	29	24.5 ± 1.1
Red & Far-red	660 & 726	9 + 1	29 (26.3 + 2.7)	24.7 ± 1.3
Tubular fluorescent lamp	6500 K[a]	1	29	25.8 ± 1.7
Darkness	-	1	0	24.6 ± 1.1

[a]Colour temperature in Kelvin.
[b]Temperature measured through the whole experiment (every hour for 16 weeks).

(Wickham et al., 2021) were employed to prepare raw data for statistical analysis. For the three experiments, the same procedure was followed: The number of regenerating explants (I) as well as the number of shoots per regenerating explant (II) were analysed in a generalised linear model (glm) with lines and cytokinins or lines and light variants and their interaction as fixed factors. Following, a deviance analysis was conducted assuming quasibinomial (I) or quasi-poisson (II) distribution. Pairwise comparisons between lines within a cytokinin or light variant and between cytokinins or light variants within a line were performed after Tukey at $p < 0.05$ using the package emmeans (Lenth, 2022).

To account for the lower number of explants in Petri dishes where contaminated explants had to be excluded, shoot regeneration percentages were calculated as weighted means and weighted standard deviations for each variant. For visualisation, bar plots were conducted using package ggplot2 (Wickham, 2016).

Pearson's correlation between the culture temperature and the regeneration percentage was analysed in the experiment 2 in order to assess the impact of the temperature measured in the light variants on the adventitious shoot regeneration of the different lines.

Results

Experiment 1: Effect of cytokinins on adventitious shoot regeneration

The aim of this experiment was to compare four cytokinins regarding adventitious shoot regeneration from primary leaf explants of 5-day-old dark-grown seedlings of four *A. hypogaea* lines (Table 1, Figure 1). Ten to 14 days after culture initiation, the primary leaf explants became green, enlarged and thickened and callus formation was observed at the proximal (basal) end of the midrib. After 21 days, shoot bud formation was initiated and the first shoots were observed 25–36 days after culture initiation (Figure S2). After the first subculture, pronounced proliferation of shoot buds was recorded, followed by the formation of first shoots (Figure S3). However, a high number of initial stages of shoot development that did not grow further into whole shoots were observed in most explants.

Both factors, the line and the cytokinin, significantly affected adventitious shoot regeneration after 16 weeks (Figure 4). The explants of three out of four peanut lines showed best regeneration frequencies on the BAP containing media (SIM1: 22.9/7.4 µM BAP and 2.3 µM Kin). Explants on SIM1 had the highest shoot regeneration frequencies with a maximum of 89.1% for 'Jimmy's Pride' being significantly higher than those recorded for MT (SIM2: 41.6%) and Zea (SIM4: 49.6%) (Figure 4). In contrast, on medium SIM3 (22.9/7.4 µM TDZ and 2.3 µM Kin), a comparably high regeneration percentage of 81.3% was reached. Meanwhile, explants of the three other lines regenerated shoots in significantly lower frequencies of 0% to 47%, with no significant differences between the media. Overall, the results showed that, the line 'Jimmy's Pride' had the highest regeneration capacity while shoot regeneration was significantly limited on explants of the line G12:PI565448.

The highest shoot number per regenerating explant of 3.1 shoots was obtained on SIM1 containing BAP for the line 'Jimmy's Pride' (Figure 5), being significantly higher than the shoot number on SIM2 (MT) with 2.2 shoots per regenerating explant. For the line G12:PI565448 only 6 explants formed one shoot each on medium SIM1, whereas on the other media no shoots were observed. The highest number of 2.8 shoots per regenerating explant was observed on SIM2 containing MT for the line G8:PI336942 and of 2.7 shoots on SIM3 containing TDZ for Dhaka (Figure 5). Considering both, the percentage of regenerating explants and the number of shoots formed per explant, medium SIM1 was found to be best suited and was therefore, selected for experiment 2 and 3.

Experiment 2: Effect of different light qualities on adventitious shoot regeneration

Based on the low regeneration frequencies as well as the low number of shoots per regenerating explant obtained in most of the peanut lines tested in experiment 1, an additional factor was varied in experiment 2 in which the effect of different light qualities was investigated to optimise shoot regeneration of three peanut lines ('Jimmy's Pride', G8:PI336942 and G12:PI565448). Therefore, a lighting module with LEDs with narrow-band light spectra applying four light variants (blue, green, green + red, red) as well as white LEDs were used as well as TFL (tubular fluorescent lamps). During the experiment, it became obvious that the same $PFD_{350\text{-}800\ nm}$ of 20 µmol $m^{-2}s^{-1}$ for the different LEDs resulted in a difference in temperatures ranging from 23.2°C under TFL to 26.2°C under blue LEDs (Table 2).

After 16 weeks of culture, the explants of 'Jimmy's Pride' under blue light had formed shoots with well-developed leaves, whereas under white LEDs and TFL pronounced callus formation was observed (Figure 6). The frequencies of shoot regeneration in this experiment were lower than 80% in all variants (even in the TFL variant, which was comparable to the SIM1 variant in experiment 1). The best shoot regeneration response and the highest shoot number per regenerating explant were obtained in the variant with blue light in all peanut lines with the maximum of 75% of explants forming shoots in line 'Jimmy's Pride' and 3.4 shoots per regenerating explant in line G8:PI336942 (Figure 7

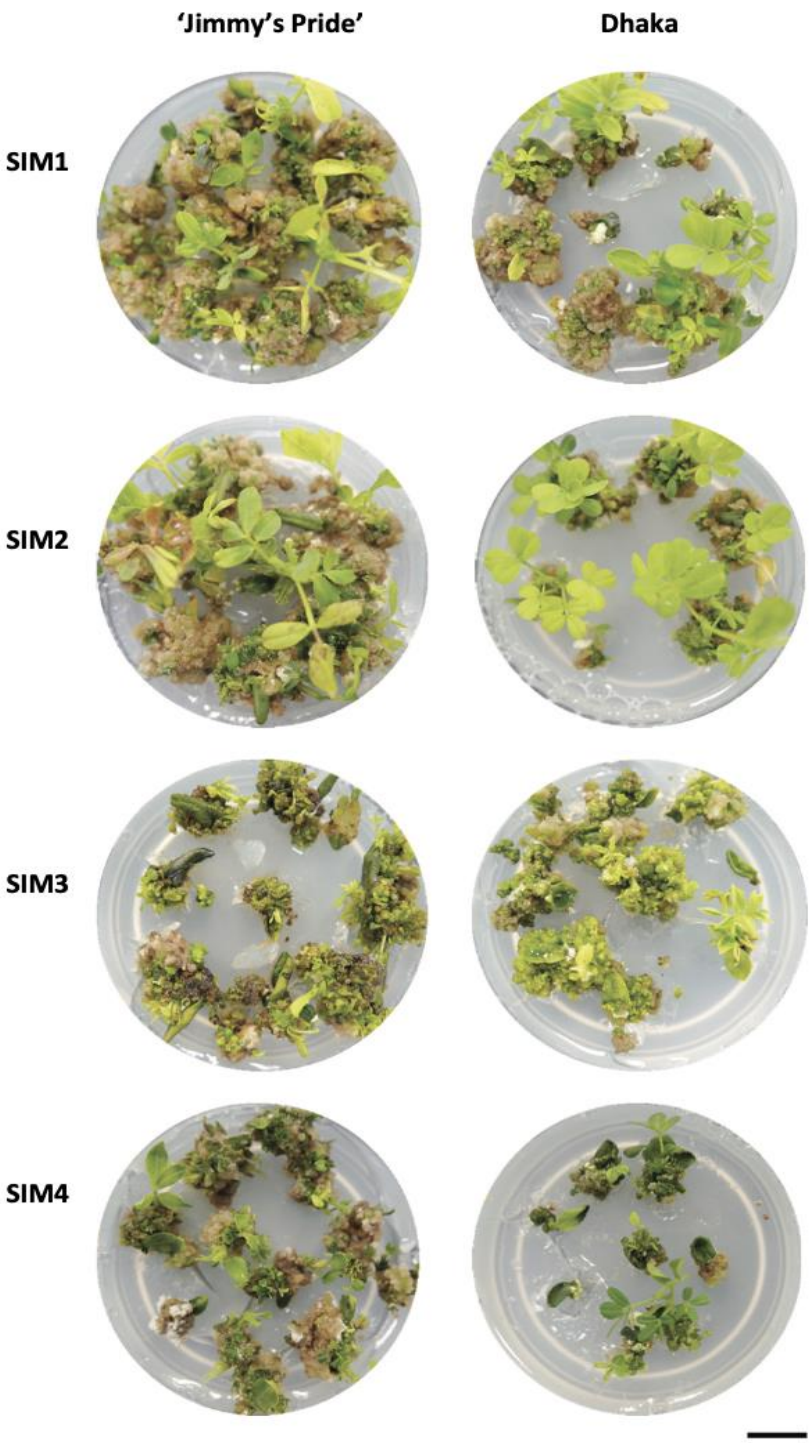

Figure 3. Effect of cytokinins on adventitious shoot regeneration from primary leaf explants of two lines of *Arachis hypogaea* ('Jimmy's Pride', Dhaka) after 16 weeks of culture on the respective shoot induction media (SIM). MS salts and vitamins were used in all media. SIM1: 22.19/7.4 (8 weeks/8 weeks) µm BAP +2.3 µm Kin, SIM2: 22.19/7.4 µm MT +2.3 µm Kin, SIM3: 22.19/7.4 µm TDZ +2.3 µm Kin and SIM4: 22.19/7.4 µm Zea +2.3 µm Kin). Bar = 1 cm.

(a,b)). Significantly lower shoot regeneration frequencies were observed with the other light variants compared to the blue light variant in all lines. In addition, the limited regeneration capacity of the line G12:PI565448 was confirmed in this experiment resulting in the lowest shoot regeneration percentages from 0% to 12.5% with 1 to 2.2 shoots per shoot forming explant.

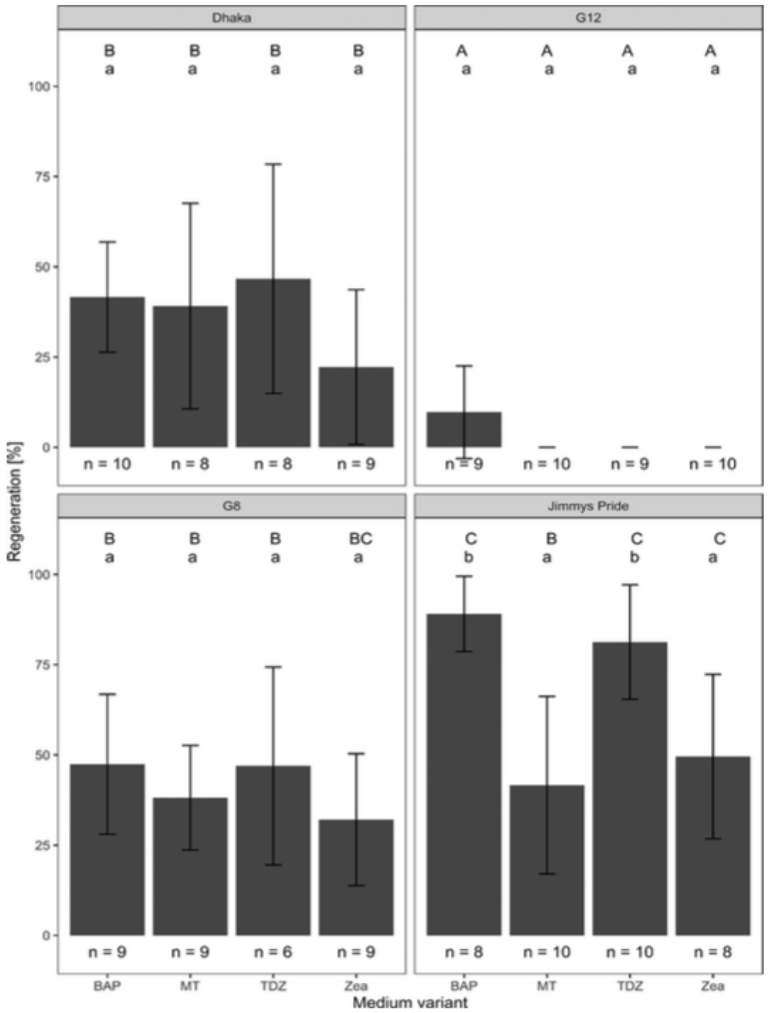

Figure 4. Adventitious shoot regeneration (percentage of explants forming shoots) of four *Arachis hypogaea* lines ('Jimmy's Pride', Dhaka, G8: PI336942 and G12: PI565448) from primary leaf explants depending on the type of cytokinin (experiment 1). *n*= number of culture vessels containing 8 explants each. MS salts and vitamins were used in all media and explants were cultivated for 16 weeks on the different media. Capital letters indicate comparisons among lines within one medium, whereas small letters indicate comparisons among media within one line. Values sharing the same letter did not differ significantly (Tukey-test $p < 0.05$).

To prove temperature effects on adventitious shoot regeneration, correlation analyses were done. The strongest positive correlation was found for the line G12 between the culture temperature and the regeneration percentage ($r = 0.94$; $p = 0.005$) while lines 'Jimmy's Pride' and G8 both also showed positive, but lower and not significant correlations with $r = 0.51$ ($p = 0.300$) and $r = 0.73$ ($p = 0.098$), respectively.

Experiment 3: Effect of different light qualities with controlled temperature on adventitious shoot regeneration

In order to avoid the overlaying effects of light quality and temperature, a bottom cooling system was installed in the lighting module for experiment 3. Thereby, the temperature was stabilised, resulting in mean temperatures of 24.5°C ± 0.3°C for all variants, except for TFL with 25.8°C (Table 3). Therefore, the effect of the different LED light variants on adventitious shoot regeneration of four peanut lines (Jimmy's pride, Dhaka, G8:PI336942 and G12:PI565448) could be investigated independent of the temperature effect. In addition to the light variants blue, green and red resulting in the best regeneration response in the experiment 2, the light variant combination red + far-red and darkness were tested due to the fact that many shoot buds were formed in experiments 1 and 2 that did not elongate.

After 16 weeks of culture, explants under red and red + far red, formed large amounts of

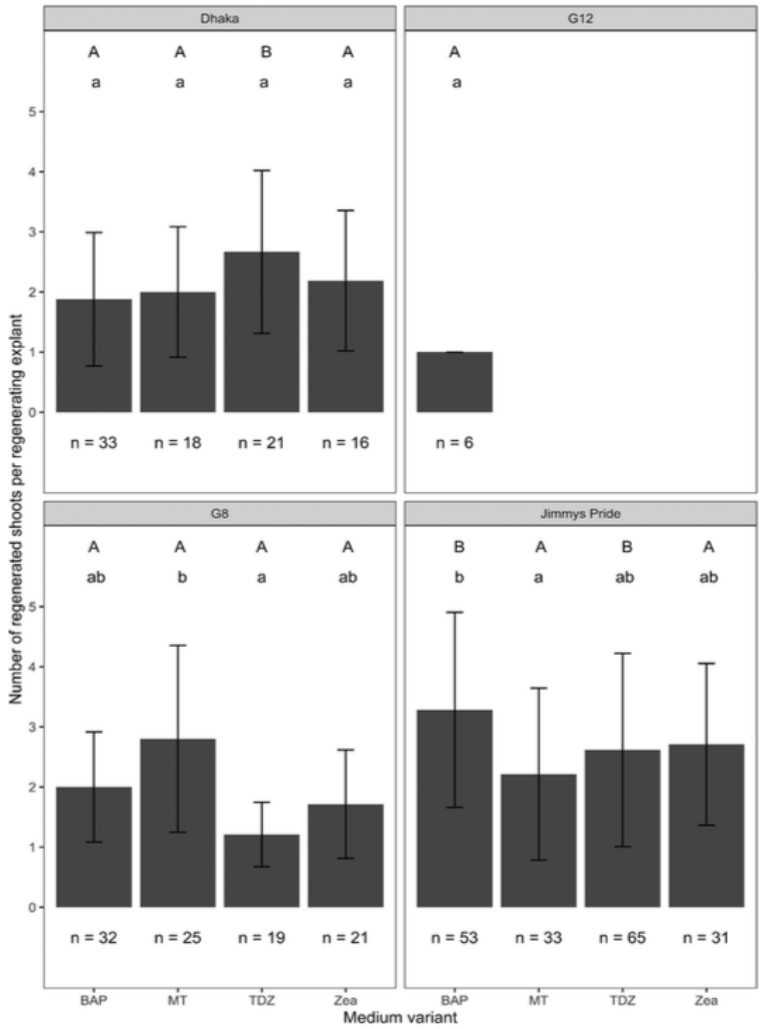

Figure 5. Shoot number per regenerating explant of four *Arachis hypogaea* lines ('Jimmy's Pride', Dhaka, G8: PI336942 and G12: PI565448) from primary leaf explants depending on the cytokinin (experiment 1). *n*= number of explants with shoot regeneration. MS salts and vitamins were used in all media and explants were cultivated for 16 weeks on the different media. Capital letters indicate comparisons among lines within one medium, whereas small letters indicate comparisons among media within one line. Values sharing the same letter did not differ significantly (Tukey-test $p < 0.05$).

whitish-grey callus, whereas explants under TFL stood out due to a high number of shoot buds and shoots (Fig. S1). In experiment 3, the frequency of shoot regeneration did not exceed 65% in any line. The highest shoot regeneration percentage of 61.1% as well as the highest shoot number per regenerating explant of 3.8 shoots, were obtained with the light variant TFL and the line 'Jimmy's Pride' (Figure 8(a,b)). The light variants red and red + far red resulted in shoot regeneration percentages which were not significantly different from TFL and were the highest in all lines (Figure 8(a)). Complete darkness led to the lowest frequencies of regenerating explants, but did not completely inhibit shoot regeneration in the lines 'Jimmy's Pride' and G8:PI336942. The superiority of the blue light variant could not be confirmed, when temperatures were regulated. Regarding the number of shoots formed per regenerating explant, no significant effect of the light variant was recorded for Dhaka and G8: PI336942. Explants of G12:PI565448 regenerated the highest number of 3.8 shoots under red light, whereas for those of 'Jimmy's Pride' TFL, red and red + far red were the variants resulting in the highest shoot numbers of 3.8, 3.2 and 3.3, respectively (Figure 8(b)). Overall, the experiments 2 and 3 proved the temperature and the line to be major factors for adventitious shoot regeneration from leaflets in peanut, but also clearly demonstrated effects of light quality.

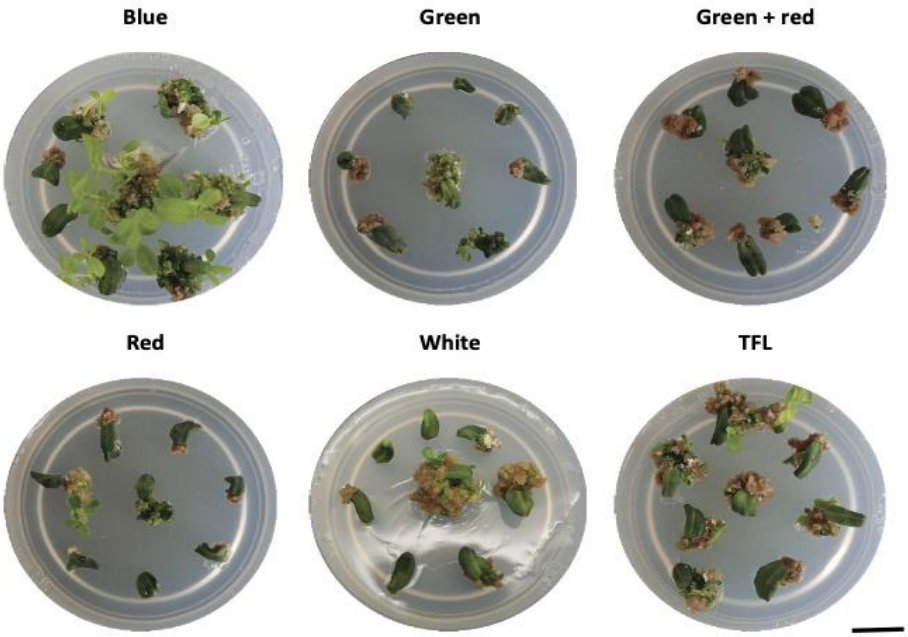

Figure 6. Effect of different spectral qualities of light on adventitious shoot regeneration from primary leaf explants of the *Arachis hypogaea* line 'Jimmy's Pride' (experiment 2). MS salts and vitamins were used in the media and explants were cultivated for 16 weeks on SIM1 (Table 1), TFL: tubular fluorescent lamps. Bar = 1 cm.

Discussion

Experiment 1: Effect of cytokinins on adventitious shoot regeneration

In the current study, an adventitious shoot regeneration protocol was successfully established for four *A. hypogaea* lines. However, the shoot regeneration response was strongly line- and cytokinin type – dependent. Moreover, relatively high standard deviations point to variability in the seedlings that were used as explant donor material. Also Matand and Prakash (2007) using leaflet explants of slightly older peanut seedlings of one Spanish type cultivar observed the type, concentration and duration of the cytokinin application to affect shoot regeneration. Immature leaflet explants of peanut were described earlier to be organogenic (Akasaka et al., 2000; Gill & Saxena, 1992; Li et al., 1994; Matand & Prakash, 2007; Tiwari & Tuli, 2009), but meta-topolin had not been tested before. The results of the present study indicated that leaflet explants of the four tested lines responded better on media SIM1 (BAP) and SIM3 (TDZ) than on SIM2 (MT) and SIM4 (Zea) (Figure 4).

The frequently applied cytokinin for shoot induction in *A. hypogaea* was thidiazuron (TDZ), which was shown to act either via stimulating endogenous cytokinin synthesis or binding to the respective cytokinin receptors in addition to blocking cytokinin oxidases and thus, cytokinin degradation (Guo et al., 2011; Mok et al., 1987). Its activity exceeds that of 6-benzylaminopurine (BAP), and zeatin (Zea) (Mok et al., 1987). It has been discussed in literature, that the different affinities and specificities of the cytokinin receptors (AHK2/3 and AHK4) for the different types of cytokinins (i.e. isoprenoid cytokinins, in our study: zeatin, aromatic cytokinins: BAP and MT and the synthetic diphenylureas: TDZ) might enable the plant to fine-tune its diverse cytokinin responses (Hwang & Sakakibara, 2006). These differences in binding affinities may explain the observed differences in shoot regeneration from leaflet explants in peanut. Because different cytokinins may have an optimal effect at different concentrations, future experiments should include concentration series.

The four lines differed significantly in their regeneration ability with 'Jimmy's Pride' giving the highest regeneration percentage of 89.1% and a mean number of 3.1 shoots per regenerating leaflet and Florunner PI565448 (G12) showing very poor regeneration (Figures 4 and 5). Genotypic differences in shoot regeneration ability were also reported in several studies before (Banerjee et al., 2007; Matand & Prakash, 2007; McKently et al., 1990; Ozias-Akins et al., 1992; Pittman et al., 1983). The effect of a specific plant growth regulators might be line-dependent and could be influenced by its interaction with endogenous growth regulators of the same class, the presence of other plant growth regulators, and also the concentration. However, also the poorly and medium regenerating lines formed callus and shoot buds, which did not develop further into shoots (Figure 3, S2 and S3). Akasaka et al. (2000) also observed regenerated shoot

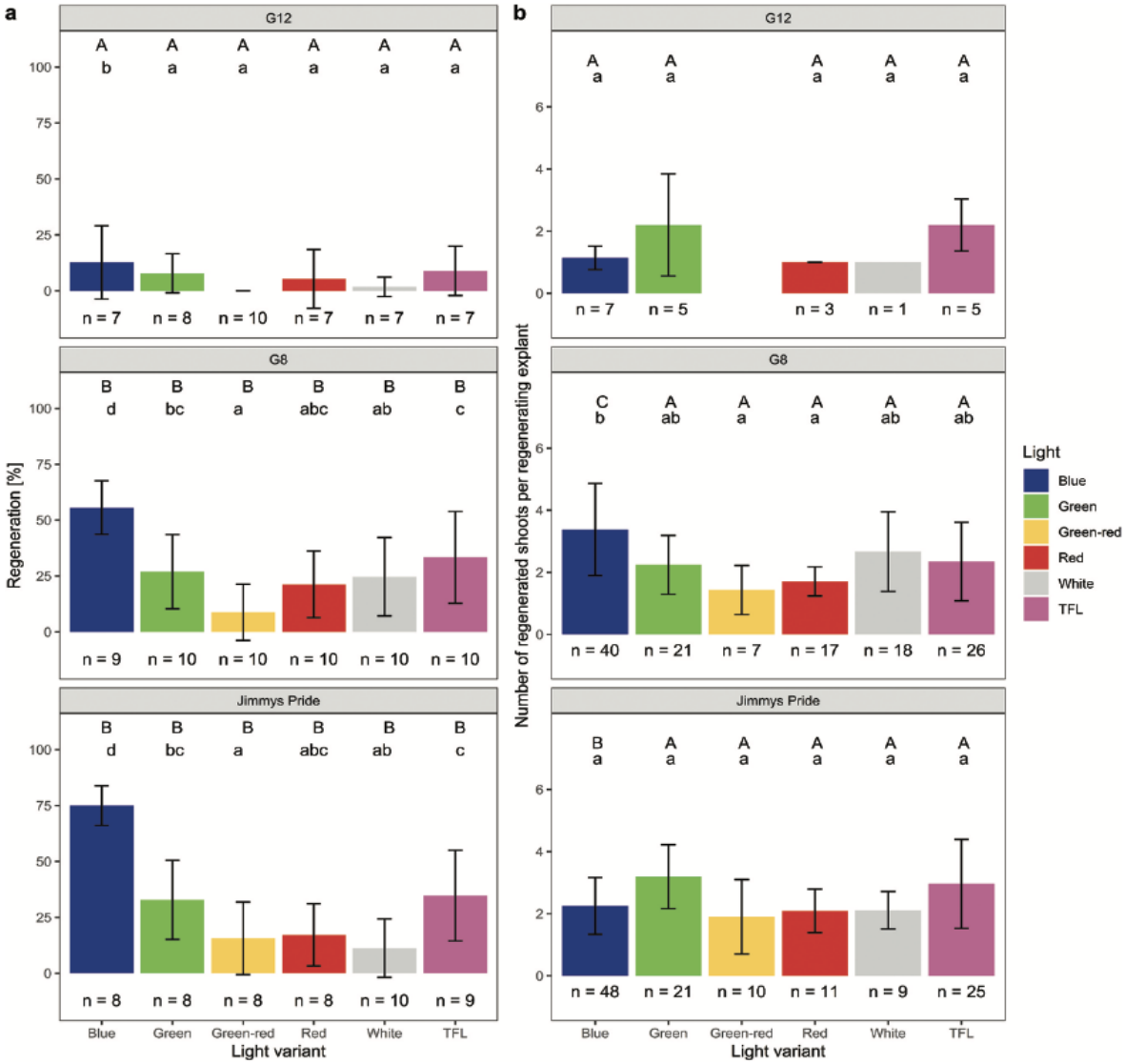

Figure 7. Adventitious shoot regeneration of three *Arachis hypogaea* lines ('Jimmy's Pride', G8: PI336942 and G12: PI565448) depending on the light quality (experiment 2). a: Shoot regeneration percentage [%] with *n*= number of culture vessels containing 8 explants each, b: Shoots per regenerating explant [count] with *n*= number of explants forming shoots. Explants were cultivated for 16 weeks on SIM1: 22.19 µM BAP +2.3 µM Kin for 8 weeks, followed by 7.4 µM BAP +2.3 µM Kin for 8 weeks. TFL: tubular fluorescent lamps. Capital letters indicate comparisons among lines within one light variant, whereas small letters indicate comparisons among light variants within one line. Values sharing the same letter did not differ significantly (Tukey-test $p < 0.05$).

buds in peanut to be blocked in development and reported severe anatomical malformations such as lacking shoot apical meristems. They could at least partly overcome the problem by reducing the TDZ concentration and/or limiting the time of exposure to TDZ. This would be important to consider in future studies applying different pulses of BAP for instance. In addition, light was investigated in this study as a factor to promote shoot elongation, especially by the far-red treatment in experiment 3.

The low shoot regeneration percentages observed in three lines could also result from the quality of the seeds used, which strongly influence seed germination and seedling vigour as recorded in soybean (Mangena, 2021). Morphogenesis in some other grain legumes is known to be very slow because of problems like the lack of response in dedifferentiated calluses (Pratap et al., 2010).

Experiment 2: Effect of different light qualities on adventitious shoot regeneration

To our knowledge, this study is the first report on the effect of different light qualities on shoot regeneration

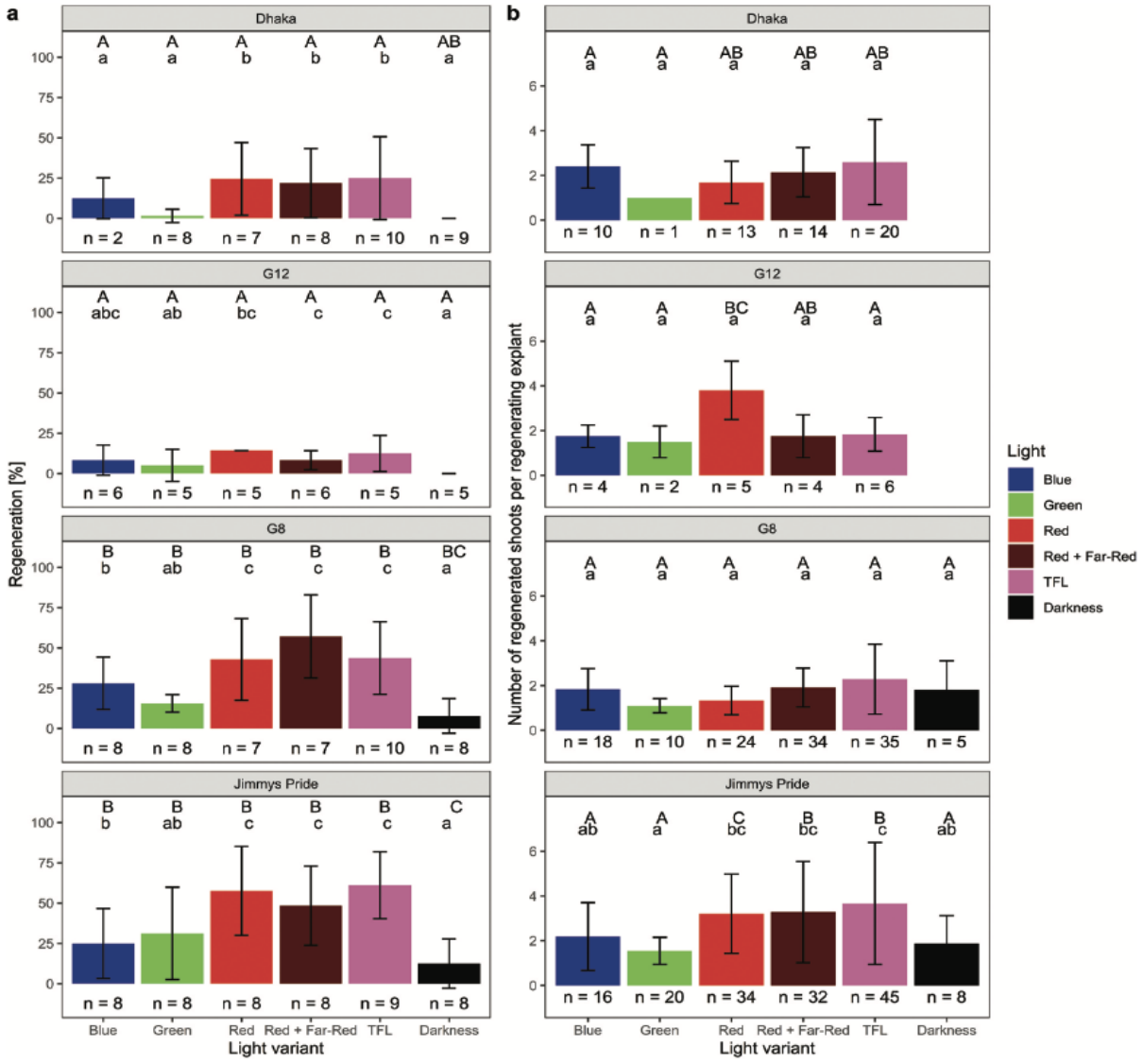

Figure 8. Adventitious shoot regeneration of four lines of *Arachis hypogaea* (Jimmy's Pride, Dhaka, G8: PI336942 and G12: PI565448) cultured under different spectral qualities of light (experiment 3). A: Shoot regeneration percentage [%] with *n*= number of culture vessels containing 8 explants each, B: Shoots per regenerating explant [count] with *n*= number of explants forming shoots. Explants were cultivated for 16 weeks on SIM1: 22.19 µM BAP +2.3 µM Kin for 8 weeks, followed by 7.4 µM BAP +2.3 µM Kin for 8 weeks. TFL: tubular fluorescent lamps. Capital letters indicate comparisons among lines within one light variant, whereas small letters indicate comparisons among light variants within one line. Values sharing the same letter did not differ significantly (Tukey-test $p < 0.05$).

of peanut. The advantage of in vitro studies in investigating morphogenetic effects of light is that photosynthesis is only of minor importance for plant growth due to the carbohydrates provided by the culture medium. In this experiment, the low shoot regeneration percentages of less than 40% under TFL was striking and can be explained by the lower culture temperature of 23°C in this setting compared to experiment 1 (Table 2; Figure 7(a)). Earlier studies showed that shoot regeneration from peanut leaf explants was strongly influenced by the culture temperature with an optimum between 28 and 35°C (Pestana et al., 1999). Also, in the present study, positive correlations were observed between the culture temperature and the regeneration percentages of the different lines indicating that the culture temperature influenced the regeneration percentages in all lines especially in line G12 with a significant and high correlation of 0.94. Therefore, it can be assumed that the significantly low shoot regeneration percentages obtained in most lines under green, green & red and red LEDs resulted from the lower temperatures of 23.4

to 24.6°C. Therefore, the standardisation of the culture temperature in all tissue culture experiments seems to be essential for the investigation of the effect of the light quality and was realised in experiment 3.

However, our results showed that shoot regeneration of the investigated peanut lines did also depend on the light quality. In this context, at the highest temperature of 26.2°C recorded under the blue and white LEDs, only blue LEDs exhibited positive effects on shoot regeneration with the highest shoot regeneration percentages of 75% in the line 'Jimmy's pride' and a mean number of 3.4 shoots per regenerating explant (Table 3; Figure 7(a,b)). Blue LEDs used in micropropagation of different plant species had positive effects on chlorophyll and carotenoid contents and chloroplast development, as well as plant growth (Bello-Bello et al., 2017; Dewir et al., 2005; Jao et al., 2005; Kim et al., 2004; Li et al., 2010, 2012; Moon et al., 2006). Blue light is perceived by the phytochrome and cryptochrome receptors of the plant: phytochromes are usually recognised as red and far-red reversible photopigments but have also absorption peaks in the blue and ultraviolet A parts of the spectrum and are involved in the regulation of plant morphology and gene expression (Quail, 2002). The cryptochromes are known as white, blue and ultraviolet A-sensitive receptors controlling several aspects of plant physiology and development such as transition to flowering, positive regulation of photomorphogenic progression, and anion channels at the plasma membrane (Folta & Childers, 2008; Folta & Spalding, 2001; Wang et al., 2001).

Light response is mediated by changes in plant hormone homoeostasis and signalling: In plants exposed to blue light, cryptochromes were observed to interact with AUX/IAA proteins, such as IAA7, IAA12 and IAA17 and to suppress the interaction between auxin and the auxin receptor TIR1 (TIR1-AUX/IAA) resulting in auxin degradation. Therefore, the cryptochromes may function as competitive suppressors of auxin signal transduction and cell elongation depending on blue light (Wang & Lin, 2020). Furthermore, according to Wang et al., (2018) and He et al. (2019) plant cryptochrome (CRY1) may interact with many brassinosteroid regulators (BRs) such as BR-INSENSITIVE 2 (BIN2), BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1) to suppress their DNA-binding activity and brassinosteroid signalling. A well-defined hormonal network that involves the biosynthesis as well as the signalling of auxin, strigolactones, and cytokinins mediates bud outgrowth: Cytokinin biosynthesis is known to be inhibited by auxin, whereas cytokinin catabolism and strigolactone synthesis are both stimulated (Demotes-Mainard et al., 2016). By this complex hormonal response, the effects of blue light may be explained, but this assumption needs to be approved by plant hormone and gene expression analyses in future studies.

Experiment 3: Effect of different light qualities with controlled temperature on adventitious shoot regeneration

Stabilising the culture temperature resulted in the determination of the effect of the light quality on adventitious shoot regeneration of the four peanut lines. Using bottom cooling of the shelves, similar temperatures in all variants were achieved with a mean temperature of 24.5°C ±0.3°C for all variants, except for TFL with 25.8°C (Table 3). The temperature variation in the custom lighting modules likely was the primary cause of the lower shoot regeneration percentages observed in all lines when compared to the experiment 1. However, the lowest seed germination percentage followed by the less developed seedlings exhibited by the line G12 might also be attributed to low seed vigour (McDonald, 2004).

Adventitious shoot regeneration was best under TFL with a shoot regeneration percentage of 61.1% and 3.8 shoots per regenerating explant in the superior line 'Jimmy's pride' (Figure 8(a,b)). The positive effects of the TFL might result from the absence of UV or cool light which are present in LEDs (Miler et al., 2019). However, since the temperature effect in our study might have been larger than that of the light quality, the TFL cannot directly be compared to the other light variants in this experiment. Remarkably, despite the lower temperatures under red LEDs and red plus far red LEDs, regeneration frequencies did not differ significantly from those under TFLs for any of the lines (Figure 8(a,b)). These results point to phytochromes to affect this organogenetic process. Previous research has shown that the phytochrome light receptors have a strong effect on auxin levels in plants by modulating both the suppressor *SUR2* and the enhancer *TAA1* of IAA biosynthesis (Halliday et al., 2009). According to Iwamoto et al. (2011), the expression of the GA biosynthesis gene *OsGA3ox2* and the ethylene biosynthesis gene *ACO1*, being involved in internode elongation in rice were affected by the three phytochromes (PhyA, PhyB, and PhyC).

In experiment 3, adventitious shoot regeneration was best for line G8 under red + far-red with a shoot regeneration percentage of 57.1% and 3.6 shoots per regenerating explant (Figure 3; Figure 8(b)). Hunter and Burritt (2004) investigated the impact of light quality on shoot organogenesis from cotyledon explants of four genotypes of *Lactuca sativa* L and found genotypic variation in the shoot regeneration under blue light. Therefore, the different responses in shoot regeneration with regard to the specific light variants could be attributed to the genetic make-up of the plant species and genotype. Shoot regeneration

was relatively low under green and blue LEDs in experiment 3 (Figure 8(a,b)). However, blue LEDs were most effective in experiment 2 when connected with a higher culture temperature indicating also an interaction of light and temperature. For upcoming experiments, combinations of red and blue LEDs should be tested at higher temperatures and could also be varied in succession, thus providing different light qualities in different developmental phases (shoot induction and shoot elongation, for instance).

Darkness did either completely inhibit (in lines Dhaka and G12) or significantly reduced shoot regeneration (Figure 8(a,b) and S1). Therefore, light represents an important factor in adventitious shoot regeneration from peanut leaflets. In agreement with this observation, Xu et al. (2018) demonstrated darkness to significantly inhibit the auxin signalling pathway, which might result from the inactivation of CRY1 and PhyB photoreceptors and thereby repressed gene expression.

Conclusions

In this study, we established a protocol for *de novo in vitro* shoot regeneration from leaflets of *A. hypogaea*, which is essential for genetic transformation and genome editing techniques. We have demonstrated that BAP was the most effective type of cytokinin to promote shoot organogenesis in peanut resulting in more than 80% of responding explants in the most responsive line. However, shoot elongation and development from shoot buds could be further optimised by reducing the exposure time of the explants to BAP as suggested by Akasaka et al. (2000). Furthermore, plants regenerated on media containing these relatively high BAP concentrations should be tested for true-to-typeness in future studies. The investigation of the effect of light quality on adventitious shoot regeneration emphasised the importance of adjusting the temperature in these kinds of experiments. Red and blue light can be used as alternatives to the traditional fluorescent lamps and further research and standardisation procedures might be necessary to investigate in more detail their potential to enhance regeneration. This will be also important for commercial laboratories and academia, since LEDs will make *in vitro* production systems more environmentally friendly and cost-effective.

Highlights

(1) A true in vitro regeneration protocol via formation of adventitious shoots was established for different *A.hypogaea* lines.

(2)The shoot regeneration ability from primary leaf explants of the different peanut lines was not only depending on the type of cytokinin but also on the genotype and the culture temperature.

(3) Fluorescent lamps could be replaced by red or blue LEDs with no negative effects on shoot regeneration.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Abbreviations

BAP	6-benzylaminopurin
IMS-PCB	Insulated metal substrate printed circuit boards
Kin	Kinetin
LED	Light-emitting diode
MT	Meta-topolin
MS	Murashige and Skoog (1962) mineral elements
NaOCl	Sodium hypochlorite
TDZ	Thidiazuron
SIM	Shoot induction media
TFL	Tubular fluorescent lamps
Zea	Zeatin

Author contributions

JA designed and performed the experiments and analysed the data. TW conceived the project. HB designed and implemented the light modules. DW performed the statistical analysis. JA wrote the manuscript with contributions of TW, HB and DW.

Data availability statement

The data that support the findings of this study are available from the corresponding author, [TW], upon reasonable request.

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5 General discussion

In this chapter, the significance of the findings of this thesis is discussed. Firstly, the relevance of the plant species' genetic make-up for adventitious shoot regeneration will be considered in chapter 5.1. We found that, different growth and developmental processes can be triggered when plants are exposed to different light qualities and highlight this in chapter 5.2. The complexity of the interaction between *Agrobacterium* and host cells is discussed in chapter 5.3, focusing on difficult-to-transform plant species. Chapter 5.4. focuses on the effect of the mutation in the mustard seed storage protein encoding gene *Bra j I*, on seed development. Finally, this general discussion is finalized with an outlook in chapter 5.5.

5.1 The importance of the genetic make-up of the plant species for adventitious shoot regeneration

The traditional method of de novo shoot induction entails tissue culture on callus induction media, followed by shoot induction on shoot induction media. This process is time consuming and shoots regenerated from callus have higher risk for somaclonal variations and loss of regeneration ability. Direct shoot regeneration, on the other hand, does not require the callus induction phase and exhibits higher regeneration potential with the production of true-to-type regenerants, which are essential for improving genetic traits in plants through genetic transformation and mass production of plantlets in breeding programs (Jung et al., 2021).

In this thesis, direct shoot organogenesis protocols for both plant species, peanut and mustard were established from explants derived from 5-days old seedlings. Taken all different lines of both species into consideration, significant variations were noticed regarding the shoot regeneration abilities of the different lines of each plant species. This conclusion is based on various experiments that examined factors such as lines, explant types and plant growth regulators in the different lines of each plant species.

The adventitious shoot regeneration rates varied quite strongly between the different peanut lines tested with the most regenerative peanut line being ‘Jimmy’s Pride’ that exhibited the most vigorous seedlings with well-developed primary leaflets and well elongated hypocotyl.

This differences in seedling vigor might result from the seed quality or the extensive genetic variability in the different peanut lines which could be attributed to the ability to mobilize and use reserve food supply from the cotyledon as well as the capacity to synthesize higher biomass during the seedling stage (Nautiyal, 2009). Moreover, it has been reported that different genotypes of peanuts may have distinct needs for plant growth regulators, resulting in notable differences in shoot regeneration (Verma et al., 2009). Lardon and Geelen (2020) referred to this as natural regenerative variability which may be caused by epigenetic, transcriptional or post-transcriptional regulation favored by key survival genes to enable fine tuning.

High cytokinin levels in the tissue culture medium activate a phosphorelay which is largely made of Arabidopsis histidine kinases (AHK2-4), phosphotransfer proteins (AHP1-5) and response regulators (RRs). This phosphorelay causes coordinated expression of shoot apical meristem (SAM) genes like *WUSCHEL* (*WUS*), *SHOOT MERISTEMLESS* (*STM*), *ENHANCED SHOOT REGENERATION* (*ESR*) *1* & *2*, and *LIGHT-SENSITIVE HYPOCOTYLS* (*LSH*) *3* & *4* (Gordon et al., 2007; Schaller et al., 2015). *WUS* is a key gene crucial for shoot apical meristem maintenance and shoot morphogenesis and it was demonstrated that type-A and type -B response regulators play a fundamental role in cytokinin signaling and were the main positive regulators of the *WUSCHEL* expression (Yadav et al., 2011). Lardon and Geelen (2020) used genome-wide association studies (GWAS) to identify a SNP that resulted in an additional response regulator (ARR) binding motif in the promoter of the beneficial *A. thaliana* Landsberg erecta (L*er*) allele. This SNP was found in a strong regenerative *Arabidopsis* line Lp2-2, which had three times higher shoot apical meristem gene *WUSCHEL* mRNA level on shoot induction medium than the poorly regenerative Col-0.

Adventitious shoot regeneration of mustard was explant- and line-specific. The results showed that, the regeneration rates varied significantly between hypocotyl and cotyledon explants in all lines. This strong variation in the shoot regeneration ability might be associated to the meristematic cells which activity could be significantly higher in the cotyledon explants than

the hypocotyl explants. This implies that, damaged tissue must be repaired through cellular regeneration, which was supposed to happen by the dedifferentiation of already mature cells, cell division to create calluses, and differentiation to create the cellular components of the new tissue (Reid and Ross, 2011). Similar observation was made by Ghimire et al. (2010) in a study investigating the direct shoot regeneration system for *Drymaria cordata* by comparing explant responses to different concentration of plant growth regulators. The study demonstrated that different explants from the same plant material act differently, suggesting that the genetic control of plant regeneration varies depending on the source of the original tissue. According to Kareem et al. (2015) cytokinin regulates the three *PLETHORA* (*PLT*) genes *PLT3*, *PLT5*, and *PLT7* that control de novo shoot regeneration in *Arabidopsis* which are upregulated independently of the shoot regeneration system. As this stem cell regulators along with *WUSCHEL-RELATED HOMEOBOX5 (WOX5)*, *SCARECROW (SCR*) are necessary to form shoot primordia by conferring the regeneration competency necessary for shoot initiation (Kareem et al., 2015; Kim et al., 2018). The hypocotyl explants of the mustard lines used in this study were less appropriate for adventitious shoot regeneration. Furthermore, histological analysis by microtome sectioning might be required to examine the meristematic cells activity along the cut edges of the hypocotyl explants. Additionally, the qPCR analysis of the expression patterns of the stem cell genes could also reveal the response level of the different genes during the culture period.

Since different explant types responded differently to particular combinations of auxin and cytokinin in *Brassica* species, the observed differential response of cotyledons and hypocotyls could also be the result of different levels of endogenous PGRs within the explants (Nguyen et al., 2021). Moreover, this could also be explained by epigenetic modification in the DNA that can be caused by external agents namely phytohormone application or stressful factors such as wounding, the secretion of ethylene or reactive oxygen species (ROS) at the early stage of shoot regeneration (Bidabadi and Jain, 2020). Therefore, the better response of the cotyledon explants to adventitious shoot regeneration may be a combination of genetic factors and adequate application of plant growth regulators.

5.2 Light quality promoting adventitious shoot regeneration

In plants, phytochromes function as a specific control mechanism for a number of physiological processes (Demotes-Mainard et al., 2016). The use of the different light qualities to improve the adventitious regeneration of different peanut lines in this study, shed more light on the effects of light quality on shoot regeneration. In this thesis, different light emitting diodes (LEDs) were used to apply specific wavelengths to the culture environment of various peanut lines. The study revealed that blue and red lights could be used to optimize adventitious shoot regeneration of peanut leaflets, and both LED lights showed similar adventitious shoot regeneration frequencies to that of conventional fluorescent lamps. However, the optimal culture temperature for peanut tissue culture, which is between 28 and 35°C, was not able to be reached in the lighting module due to the use of various LEDs at the same $PFD_{350\text{-}800nm}$, which caused a variance in temperature (Chapter 4). This temperature variation in the lighting module strongly affeceted the regeneration frequencies of the leaflet explants in all peanut lines.

It is known that phytochromes (phy) and cryptochromes (cry) are essential for photomorphogenesis as they are involved in several plant hormonal pathways. There is now substantial evidence that plants respond to light signals in a highly specific manner. The photoreceptor phyB functions as a light-reversible molecular glue by promoting the degradation of target proteins via the improvement of E3 ubiquitin ligase binding which is mediated by the Skp, Cullin, F-box containing complex (SCF complex) (Shi et al., 2016). This mechanism is involved in the signal transduction of several plant hormones namely **ethylene** through the binding of the transcription factor EIN3 (Ethylene Insensitive 3) to its E3 ligases EBF1/EBF2, resulting in EIN3 degradation (Shi et al., 2016); **auxin** through the binding of TIR1 F-box protein and its substrate Aux/IAA proteins which cause the repressors AUX/IAA to be ubiquitinated (Tan et al., 2007); **jasmonic acid**, through the binding of JAZ proteins to the F-box protein COI1, resulting in the poly-ubiquitylation and further degradation of JAZ (Sheard et al., 2010); and **gibberellic acid**, through the binding of DELLA proteins and its F box protein leading to the ubiquitination of DELLA (Murase et al., 2008).

Throughout the light spectrum, plants absorb varying amounts of light energy. Even though the intensity (in terms of µmol m^{-2} s^{-1}) of each light source was controlled, blue light's higher energy and shorter wavelength may have different effects on plants than red light's lower energy and longer wavelength (Kwon et al., 2015). According to Wei et al. (2020), red LEDs significantly upregulated the expression of key genes namely *WOX5*, *LBD16*, *LBD18*, and *PLT3* involved in the formation of shoot primordia, the promotion of the callus pluripotency and the development of roots. Red light promotes leaf expansion, hypocotyl elongation and root development, whereas blue light inhibits hypocotyl elongation and stimulates biomass production (Johkan et al. 2010; Park and Kim, 2010). When combined, red and blue light promote plant growth, namely early stem elongation and leaf growth (Folta, 2004; Johkan et al. 2012). The beneficial effect of combined red and blue LEDs may emerge from the synergetic interactions between cryptochromes and phytochromes, which in turn promote or repress stem elongation in various ways in various species. Consequently, further investigation of the effect of the combined red and blue lights at the optimal regeneration temperature might be necessary to improve the shoot regeneration system of peanut leaflet explants.

On the other hand, high shoot regeneration rate was as well attained when peanut leaflet explants were cultured under the combination of red plus far red lights. It was suggested that in *Arabidopsis* leaves, the low red to far red (R/FR) ratio light modulates the activity of the phytochrome B by regulating the expression of the PIF7 (phytochrome interacting factor 7) and therefore promoting the interaction of PIF7 and phytochrome B leading to the expression of flavin monooxygenase (YUC), a rate-limiting enzyme in the auxin synthesis pathway (Yang et al., 2020). According to Demotes-Mainard et al. (2016), plant developmental processes namely bud outgrowth, stem elongation and leaf growth are promoted by low R/FR ratio light. Additionally, under low R/FR ratio light, Yang et al. (2020) reported stem elongation and increase of biomass production and leaf area of soybean plantlets whereas Kurepin et al. (2007) also observed maximum stem elongation and reduction in ethylene levels in sunflower plantlets under low R/FR ratio light. In this study, high R/FR (9/1) ratio light was used therefore, further investigation might be necessary to also evaluate the impact of the low R/FR ratio light.

Moreover, the use of a specific wavelength at different adventitious shoot regeneration phases of the peanut leaflet explants could as well be examined.

5.3 The complexity of the interaction between *Agrobacterium* and host cells makes the genetic transformation challenging

The genetic transformation experiments conducted in this study were quite challenging considering the low transformation efficiency obtained with the mustard lines as well as the lack of transgenicity exhibited by the regenerated peanut shoots. *Agrobacterium*-mediated genetic transformation involves several steps starting with the detection and sensing a wounded host cells by a virulent *Agrobacterium* and ends with the integration and the expression of the T-DNA in the transformed cell's genome. This process is influenced by a number of factors namely the bacterial strains, plasmids, the explant wounding, the tissue culture environment, the media for explant culture, the duration of co-cultivation, the selective marker, and the competency of target plant tissue for infection and regeneration (Karami et al., 2009).

From the different transformation experiments conducted with more than 7000 explants including embryo axis; de-embryonated cotyledons and leaflets, obtained from 5 days old seedlings of the peanut lines 'Jimmy's Pride', 'Dakha' and Florunner PI565448 (G12) (Table 3), no transgenic shoots were obtained. For the genetic transformation, *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vectors PLC27 (carrying a green fluorescence protein and hygromycin resistance) and pEGFP (carrying an enhanced green fluorescence protein and kanamycin resistance) as well as three CRISPR/Cas9 binary constructs PLC68 (carrying eight sgRNAs and hygromycin resistance), PLC71 (carrying eight sgRNAs and kanamycin resistance) and PLC8888 (carrying four sgRNAs and kanamycin resistance) were provided by Prof. Jens Boch and Dingbo Zhang from the institute of Plant Genetics at Leibniz University Hannover, Germany. The three CRISPR/Cas9 binary constructs target the conserved region of the two *Ara h 1* homoelogs in the genome of peanut (*Ara h 1A*

and *Ara h 1B*) to introduce deletions or insertions at the cleavage sites that can lead to frame-shift mutation of *Ara h 1A* and *Ara h 1B*.

Table 3. Overview of the transformation experiments carried out on peanut lines used in chapter 4 ('Jimmy's Pride', 'Dhaka' and PI565448 (G12)) with GFP (PLC 27 and pEGFP) and CRISPR/Cas9 constructs (PLC 68, PLC 71 and PLC 8888), including selection agent, explant type and the number of explants used, DEC: De-Embryonated Cotyledon.

Experiment n°	Line	Plasmid	Explant type and number			Selection
T1	Jimmy's Pride	PLC27	Embryo: 100	DEC: 200	Leaflets: 800	Hygromycin
T2	Jimmy's Pride	PLC68	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
		PLC71	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
T3	Jimmy's Pride	PLC68	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
		PLC71	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
T4	Jimmy's Pride	PLC27	Embryo: 30	DEC: 60	Leaflets: 240	Hygromycin
		PLC68	Embryo: 30	DEC: 60	Leaflets: 240	Hygromycin
T5	Jimmy's Pride	PLC71	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
		PLC68	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
		PLC8888	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
T6	G12: PI565448	PLC71	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
		PLC8888	Embryo: 50	DEC: 100	Leaflets: 400	Hygromycin
T7	Dakha	pEGFP	Leaflets: 100			Kanamycin
		PLC68	Leaflets: 100			Hygromycin
		PLC8888	Leaflets: 100			Hygromycin
T8	Dakha	pEGFP	Leaflets: 100			Kanamycin
		PLC71	Leaflets: 100			Kanamycin
		PLC8888	Leaflets: 100			Kanamycin
T9	Dakha	PLC71	Leaflets: 100			Kanamycin
		PLC8888	Leaflets: 100			Kanamycin
T10	Dakha	PLC71	Leaflets: 100			Kanamycin
		PLC8888	Leaflets: 100			Kanamycin

In the different transformation experiments conducted, shoots could be regenerated from all treated explants in all lines, the transgenicity of which could not be confirmed through PCR.

The lack of transgenicity might result from several factors including the efficiency of the interaction of *Agrobacterium* with cells of the target tissue, the competency of target plant tissue for infection, the regeneration capacity of the transformed cells of the target tissue and the selection time of the transformed cells (Karami et al., 2009; Chen et al., 2015). There has been limited advancement in improving legumes using transgenic methods because of their recalcitrant nature to in vitro regeneration, which is particularly pronounced in the case of peanuts. According to Chen et al. (2015), only a few genotypes of peanut are amenable to regeneration following *Agrobacterium*-mediated transformation which represents a significant limitation to the use of modern breeding techniques. However, several studies have reported successful *Agrobacterium*-mediated transformation of peanut using *GUS* reporter gene (Cheng et al., 1996 and Li et al., 1997); viral genes (Bag et al., 2007 and Tiwari et al., 2008) and RNAi technology (Dodo et al., 2008).

Plant genetic transformation begins with the induction of *Agrobacterium vir* region by particular host signals, often small phenolic and specific monosaccharide molecules (Tzfira et al., 2004). The *Agrobacterium* strain LBA4404 used in this study is considered as one of the most effective strains for the genetic transformation of legumes (Chen et al., 2015). According to Suzuki et al. (2001), the effectiveness of LBA4404 might be attributed to the super binary vector pTOK233, which contains *virB*, *virC* and *virG* genes generated from the 'supervirulent' Ti-plasmid; pTiBo542. Consequently, the bacteria and their virulence are at least not likely the cause of the lack of transgenicity in this study. Moreover, in this study, a successful *Agrobacterium*-mediated transformation protocol of different lines of *B. juncea* was established using the strain LBA4404.

However, in *A. tumefaciens*-mediated transformation, the plant is involved in a number of processes, including the bacterium attachment to the plant surface, the transfer of the T-DNA and virulence proteins through the plant cell wall and plasma membrane to cytoplasm, the transport of the T-strand/protein complexes through the cytoplasm and the nuclear targeting of these complexes, the integration of the T-DNA into the host genome and the subsequent

expression of the T-DNA encoded genes (Zhu et al., 2003). Among all steps involved in the genetic transformation process, T-DNA integration is likely the one that depends the most on host cellular functions. According to Tzfira et al. (2004), the host DNA repair machinery is the only mechanism involved in T-DNA integration, which transforms the T-strand molecule into double-strand molecule T-DNA intermediates, to identify these molecules as damaged DNA fragments, and insert them into the host genome.

According to Mysore et al. (2000), the *Arabidopsis* gene *HTA1* is believed to be involved in T-DNA integration step of *Agrobacterium*-mediated transformation since its overexpression in wild-type *Arabidopsis* plants markedly increased their susceptibility to *Agrobacterium* infection. Similar to this, the overexpression of the *VIP1* gene, a plant protein required for T-DNA nuclear import, greatly improved the susceptibility of tobacco plants to *Agrobacterium*-mediated genetic transformation (Tzfira et al., 2004). *Agrobacterium* infection could also be improved by the overexpression of the VirB2-interacting protein (BTI), a plant protein that has been found to interact with the *Agrobacterium* T-pilus protein VirB (Hwang et al., 2004).

Moreover, upon *Agrobacterium* infection, the plant defense machinery is activated through regulating protein activity, enhancing reactive oxygen species (ROS) accumulation and phytohormone synthesis, namely salicylic acid and jasmonic acid (Bigeard et al. 2015). This triggers the expression of many genes resulting in secretion of H ions, phenolics and carbohydrates, to heal the cell damage at the wound site (Lacroix and Citovsky, 2013), after which callose is deposited at the cell wall to strengthen the composition of the wall and stop pathogen entry into the plant (Janda et al. 2019). In this work, Silwet L-77 was used as a surfactant that reduces tension and thereby allows exogenous microorganism to enter plant through natural openings or wounds (Liu et al., 2008). Furthermore, according to Tiwari et al. (2022), in response to pathogen attack, maize seedlings secrete chemicals such as DIMBOA (2,4-dihydroxy7-methoxy-2H-1,4-benzixazin-3(4H)-one) and MDIBOA (2-hydroxy-4,7-dimethoxybenzoxazin-3-one), which inhibited *Agrobacterium* growth and prevented virulence induction.

On the other hand, the regeneration capacity of the transformed cells is determined by the ability of the transgenic cells to undergo dedifferentiation and acquire the pluripotent stem cells (PSCs). It is suggested that the cell cycle stage at the time of transformation is essential to acquire pluripotency as the dividing cells are the most effective targets for transgene nuclear integration and the frequency of the S and G2 stages of the cell cycle has been strongly correlated with the regeneration of transgenic shoots (Delporte et al., 2012; Sabbadini et al., 2019). In addition, the tissue-culture media components and culture conditions have a significant impact on the frequency of callus induction and subsequent regeneration into transgenic plants. Lowe et al. (2003) found that overexpression of the *Arabidopsis* regulators LEAFY COTYLEDON 1 (LEC1) and LEC2 resulted in the improvement of the transformation efficiency in maize and wheat whereas Lowe et al. (2016) reported similar results when overexpressing of the combined BABYBOOM (BBM) and WUSCHEL (WUS) in maize, rice and sorghum. Based on the different factors involved in the interaction between *Agrobacterium tumefaciens* and the host cells, it is suggested that, the lack of transgenicity in the *Agrobacterium*-mediated transformation of peanut observed in this work might result from the failure of the bacteria to infect the targeted plant tissue or the transformed peanut cells could be incompetent for regeneration. Moreover, since the selection of the transgenic shoots in this work started two weeks after the end of co-culture, it could be that the transformed cells were overgrown by the non-transformed cells as with mustard, the selection started immediately after the end of the co-culture.

5.4 Bra j I mutation as causal agent of abnormal seed development

The Bra j I mutation obtained in some transgenic mustard lines might have affected seed development, as evidenced by the anomalies in seed shape, low seed weight and poor seed germination rates found in some lines expressing the mutation (Chapter 2). Based on the significant differences in these traits observed between some transgenic lines expressing the mutation and the wild types, the analysis of the early embryogenesis through the ovule growth was required. Therefore, young floral buds from two transgenic lines (22 and 32, chapter 2) that were found to have frame shift mutations in all four *Bra j I* alleles as well as the wild type

were selected, carefully emasculated and controlled pollinated with pollen from different flowers of the same plant. The dynamic observation was conducted at six time points i.e., 3, 7, 10, 15, 17 and 20 days after flowering (DAF). To preserve the genetic identity of the samples of the different lines, the selected samples were protected with isolation bags. At each stage, twenty flowers/pods were collected from each plant and ovaries were isolated and prepared for differential interference contrast (DIC) microscopy. The detailed procedure for DIC microscopy was described by Braun and Winkelmann, (2016).

The examination of the ovule development revealed that in the majority of the selected samples from both transgenic lines, no embryos could be found. Furthermore, a significant number of ovules from both transgenic lines turned brown already at 7 DAP and were completely brown at 15 DAP. This might be the result of unfertilized ovules or ovules that were aborted before the zygotic embryo development was activated.

After pollination, ethylene level rises in ovules are thought to play a key role in the regulation of early ovule development (Wang et al., 2021). In transgenic tobacco plants, De Martinis and Mariani, (1999) found that suppressing the gene expression of the ethylene-forming enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) prevented ovules from completing megasporogenesis and producing embryo sacs. Therefore, pollen tubes entering the ovary failed to grow toward the immature ovule. Moreover, it has been demonstrated that genes essential for the development of the female gametophyte and the ovule, including *AINTEGUMENTA* (*ANT*), encode putative transcription factors that are members of the same family as the ethylene-responsive element binding proteins (EREBPs) (De Martinis and Mariani, 1999). On the other hand, the overaccumulation of ethylene-insensitive 3 (EIN3) which regulates the majority of downstream target genes and most ethylene responses (Chang et al., (2013) in the synergid cells might prevent pollen tube attraction and could be crucial in the connection between ethylene overaccumulation and ovule abortion (Wang et al., 2021). According to Wang et al. (2021), ethylene signaling increases the transcription of the gene for ethylene-insensitive 3-like (EIL1), and EIL1 then promotes the expression of the gene SENESCENCE-ASSOCIATED CYSTEINE PROTEINASE 1 (Cysp1), which is involved in the

senescence of unfertilized ovules. As a result, it is also possible that the Bra J I mutant lines may experience changes in the gene expression levels which could be related to the regulation of some genes that lead to ovule abortion. The *Cysp1* gene was as well identified in *Brassica napus*, which is an integument-specific cysteine proteinase of the ovule and linked to programmed cell death (PCD) of the inner integument (Wan et al., 2002).

Due to time constraints and the presence of yellowish flowers/pods with brown ovules, particularly in line 22, it was not possible to thoroughly examine the ovules at different stages of embryogenesis, so only exemplary impressions were documented (Figure 1).

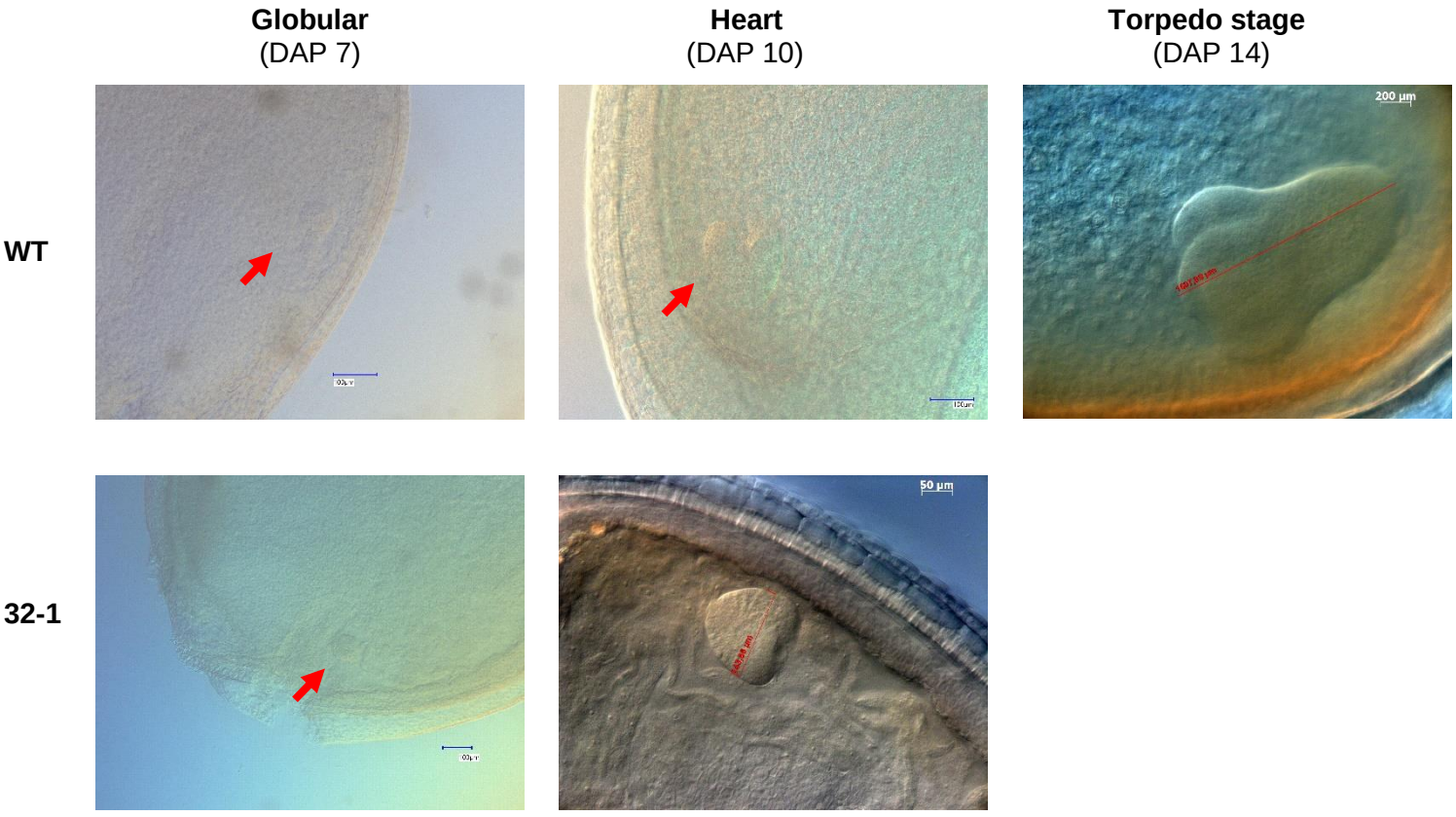

Figure 1. Embryonic development of the *Brassica juncea* line CR2664. DAP: day after pollination, WT: wild type, 32-1: plant from the first inbreeding generation of the transgenic line 32.

Nevertheless, in comparison to the wild type, overall, no abnormalities from normal embryogenesis were found in the early stages that were investigated. Brown ovules as well as ovules with or without embryo at different developmental stages might be used in histological analysis by microtome sectioning to examine how the *Bra J I* mutation affected the embryogenesis of the transgenic lines. Additionally, it may be important to compare the protein

homeostasis between the Bra J I mutants and the wild type through proteomic analysis in order to determine how the Bra J protein interacts with other proteins and understand the implications of the Bra J I mutation in seed development. A gene expression analysis of the major genes involved in the biosynthesis of the sesquiterpenoid hormone abscisic acid (ABA) including *ABA-DEFICIENTS4* (*ABA4*) and *NEOXANTHIN-DEFICIENT1* (*NXD1*), in mustard seeds may also be necessary because ABA is crucial for seed development (Sano and Marion-Poll, 2021).

5.5 Outlook

Future studies could focus on characterizing the expression level of the genes involved in the positive regulation of shoot regeneration namely *WUSCHEL-RELATED HOMEOBOX5 (WOX5)*, *SCARECROW (SCR*) and the three *PLETHORA* (*PLT*) genes *PLT3*, *PLT5*, and *PLT7* in the various explants of both plant species on the shoot induction medium in order to identify the genetic factors favoring the shoot regeneration abilities of the superior lines of both plant species. In parallel, more investigation should be conducted to identify the distinct needs for plant growth regulators in all lines and their optimal application to be associated with the physiological stages of the plants namely the shoot induction and elongation.

The culture temperature of the home-made light module should be adjusted to the optimal culture temperature of shoot regeneration in peanut, which could reveal the adequate light regime necessary for shoot regeneration. In addition, the ethylene production of the peanut explants at the early stage of shoot induction could also be determined for subsequent improvement of shoot regeneration efficiency using silver nitrate, a well-known ethylene inhibitor.

To find out whether the activation of the peanut defense system restricts *Agrobacterium* development and prevents the induction of virulence, further research on the metabolites secretion from the peanut explants after wounding is necessary. Based on the recalcitrant nature of peanut to plant regeneration and genetic transformation, the supplementation of antioxidants such as $_{L}$-Cysteine, glutathione, DL- α- tocopherol and selenite to the co-culture media could decrease the oxidation stress generated from the cut surface of the peanut explants and thereby minimize the effects of leached phenolics. Moreover, more investigation should be conducted to establish a pre-culture period of the peanut explants prior to *Agrobacterium* infection and to determine the optimum *Agrobacterium* optical density for an effective genetic transformation of peanut. Since we observed abnormalities on the seeds shape, seed weight, seed production and low seed regeneration with some edited lines of

mustard, further investigation of the embryogenesis is necessary to elucidate the effect of mutation on the seed development.

6 Conclusion

In this thesis, the influence of the genetic make-up on the shoot regeneration as well as the *Agrobacterium*-mediated genetic transformation of different lines of *B. juncea* and *A. hypogaea* was revealed. Adventitious regeneration systems for different lines of *B. juncea* and *A. hypogaea* were successfully established which represents a milestone for applying modern breeding techniques such as CRSIPR/Cas9 in both plant species. Moreover, an optimized regeneration protocol was developed for the European lines of mustard, that were thought to be very recalcitrant to in vitro regeneration. For the first time, the allergen Bra J I, the most predominant seed storage protein in both European and Indian lines of mustard was successfully engineered using the gene editing method CRISPR/Cas9 and transgenic plants were obtained carrying deletions and frameshift mutations. On the other hand, the interaction between light quality and the plant photoreceptors of the different peanut lines showed the importance of specific wavelengths in shoot morphogenesis. In contrast to mustard, the main challenge experienced with the genetic transformation of peanut was the lack of transgenicity in the regenerated shoots of all peanut lines tested. This underlines the complexity of the interaction between the *Agrobacterium* and the host cells of different plant species, which needs further investigations in order to understand and overcome the lack of transgenicity.

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Supplemental materials for chapter 2

Supplemental Table S1. Number of explants per line and medium used to investigate the effect of plant growth regulators on adventitious shoot regeneration from cotyledon explants of eight lines of *Brassica juncea* (six European lines: 'Terraplus', 'Terratop', 'Terrafit', 'Energy', SFB18/1 and SFB22/15 and two Indian lines: CR2649 and CR2664) (Experiment 1). MS salts and vitamins were used in all media and explants were cultivated for 4 weeks on the different media. SIM 1: 2.22 µM BAP + 0.49 µM IBA, SIM 2: 4.44 µM BAP + 0.49 µM IBA, SIM 3: 8.88 µM BAP + 0.49 µM IBA, SIM 4: 17.76 µM BAP + 4.92 µM IBA.

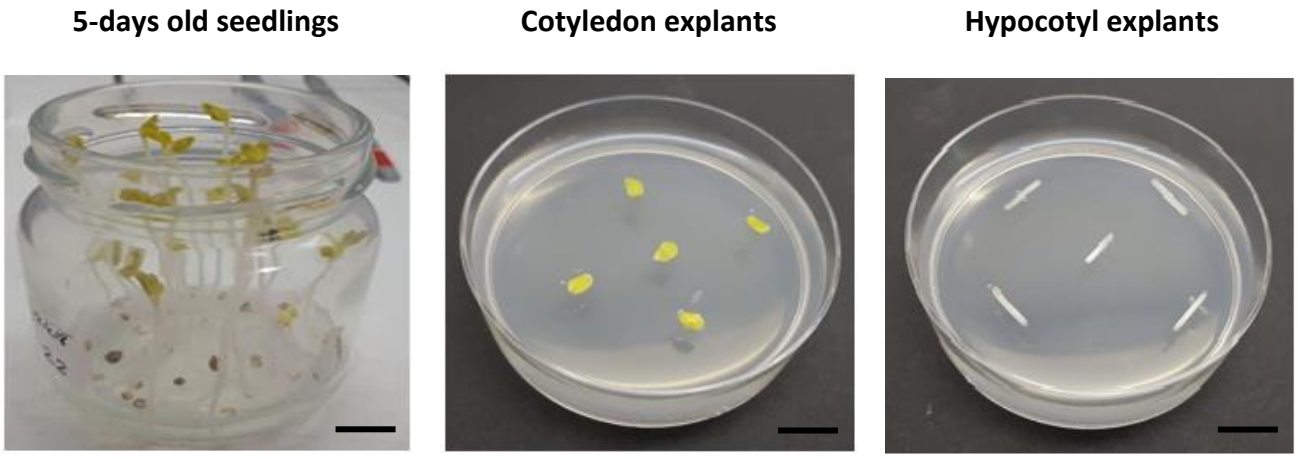
	Number of cotyledon explant evaluated for the shoot regeneration [%] numbers in brackets refer to the number of replicates (Petri dishes)				Number of explants with shoots			
Line	SIM 1	SIM 2	SIM 3	SIM 4	SIM 1	SIM 2	SIM 3	SIM 4
'Terraplus'	40 (8)	60 (12)	50 (10)	50 (10)	5	16	9	15
'Terratop'	55 (11)	55 (11)	55 (11)	55 (11)	17	25	14	26
'Terrafit'	50 (10)	55 (11)	49 (10)	60 (12)	10	17	8	21
'Energy'	45 (9)	50 (10)	45 (9)	55 (11)	19	11	9	23
SFB18/1	47 (10)	48 (10)	55 (11)	60 (12)	7	10	9	18
SFB22/15	50 (10)	55 (11)	50 (10)	55 (11)	11	13	16	19
CR2649	40 (8)	60 (12)	55 (11)	50 (10)	15	21	15	20
CR2664	50 (10)	55 (11)	55 (11)	50 (10)	15	21	22	23

Supplemental Table S2. Number of explants per line and medium used to investigate the effect of silver nitrate ($AgNO_3$) and BAP on adventitious shoot regeneration from the cotyledon explants of five lines of *Brassica juncea* (three European lines: 'Terratop', 'Energy' and SFB22/15 and two Indian lines: CR2649 and CR2664) (Experiment 2). MS salts and vitamins were used in all media and explants were cultivated for 4 weeks on the different media. SIM 4: 17.76 µM BAP + 4.92 µM IBA, SIM 5: 8.88 µM BAP + 5.37 µM NAA+ 9.95 µM $AgNO_3$, SIM 6: 8.88 µM BAP + 0.49 µM IBA+ 9.95 µM $AgNO_3$

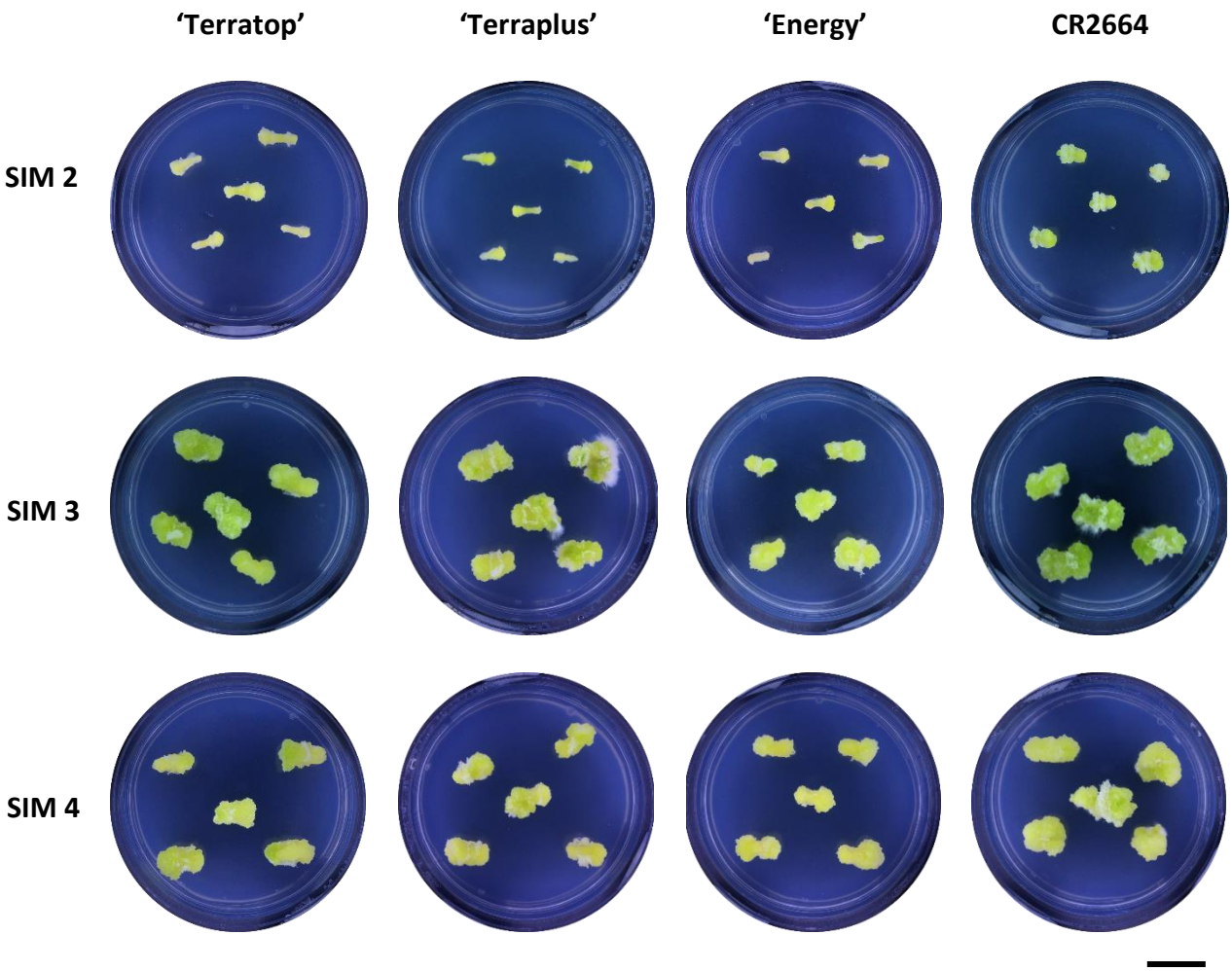
Line	Number of cotyledon explant evaluated for the shoot regeneration [%] numbers in brackets refer to the number of replicates (Petri dishes)			Number of explants with shoots		
	SIM 4	SIM 5	SIM 6	SIM 4	SIM 5	SIM 6
'Terratop'	55 (11)	60 (12)	55 (11)	21	43	36
'Energy'	55 (11)	60 (12)	60 (12)	16	35	24
SFB22/15	60 (12)	50 (10)	55 (11)	22	35	22
CR2649	55 (11)	60 (12)	55 (11)	27	39	36
CR2664	55 (11)	60 (12)	60 (12)	36	54	39

Supplemental Table S3. Number of explants per line and variant used to investigate the effect of ACC and $AgNO_3$ on adventitious regeneration from cotyledon explants of four European lines of *Brassica juncea* ('Terratop', 'Terraplus', 'Energy' and SFB22/15) (Experiment 3). MS salts and vitamins were used in all media variants and explants were cultivated for 4 weeks on the different media. Control: 8.88 µM BAP + 5.37µM NAA, ACC: 8.88 µM BAP + 5.37µM NAA + 500 µM ACC, $AgNO_3$(SIM 5): 8.88 µM BAP + 5.37µM NAA + 9.95 µM $AgNO_3$

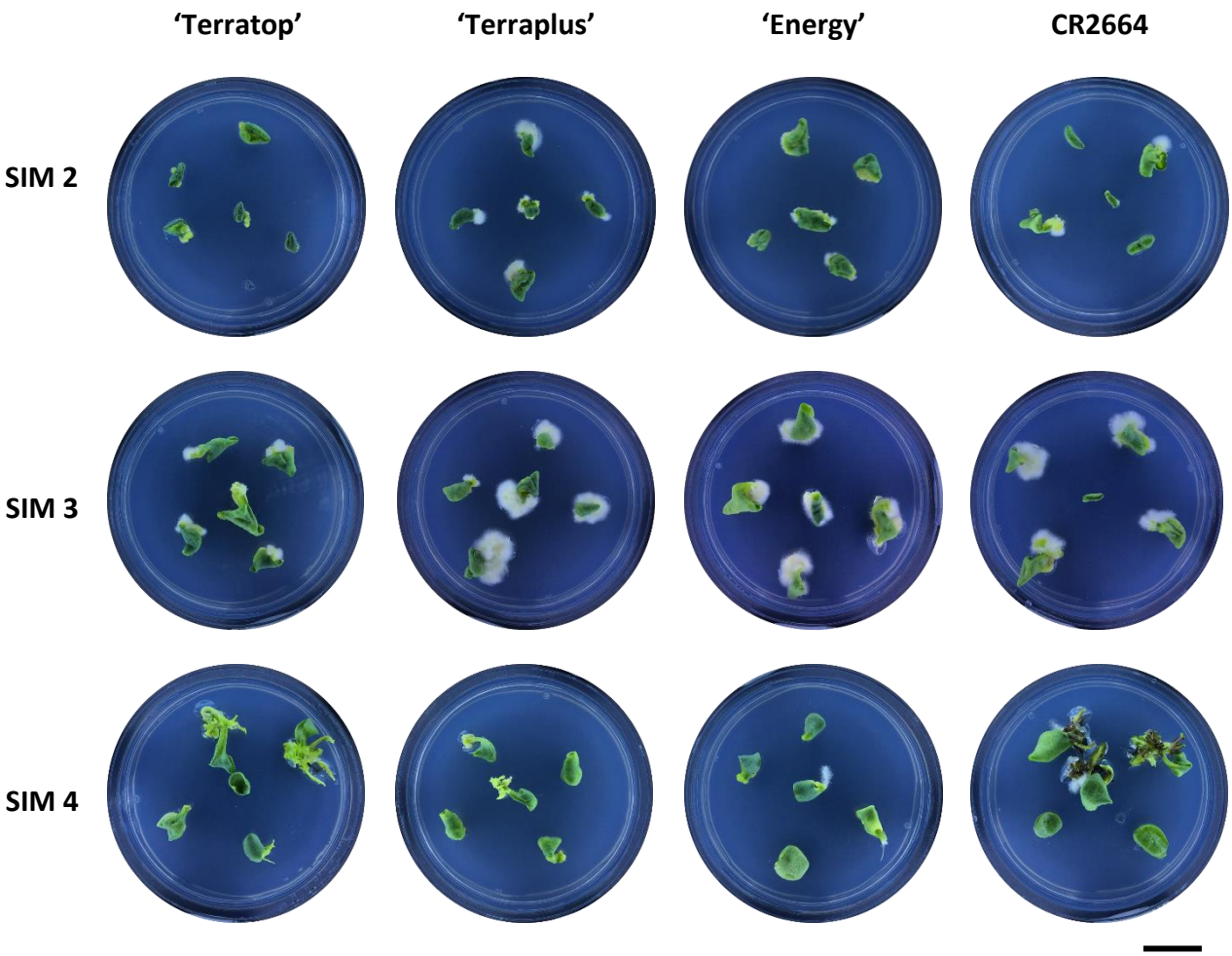
	Number of cotyledon explant evaluated for the shoot regeneration [%] numbers in brackets refer to the number of replicates (Petri dishes)			Number of explants with shoots		
Line	Control	ACC	$AgNO_3$	Control	ACC	$AgNO_3$
'Terratop'	59 (10)	59 (10)	59 (10)	32	3	45
'Terraplus'	59 (10)	59 (10)	57 (10)	8	1	44
'Energy'	58 (10)	59 (10)	59 (10)	9	6	40
SFB22/15	58 (10)	58 (10)	54 (9)	14	4	14



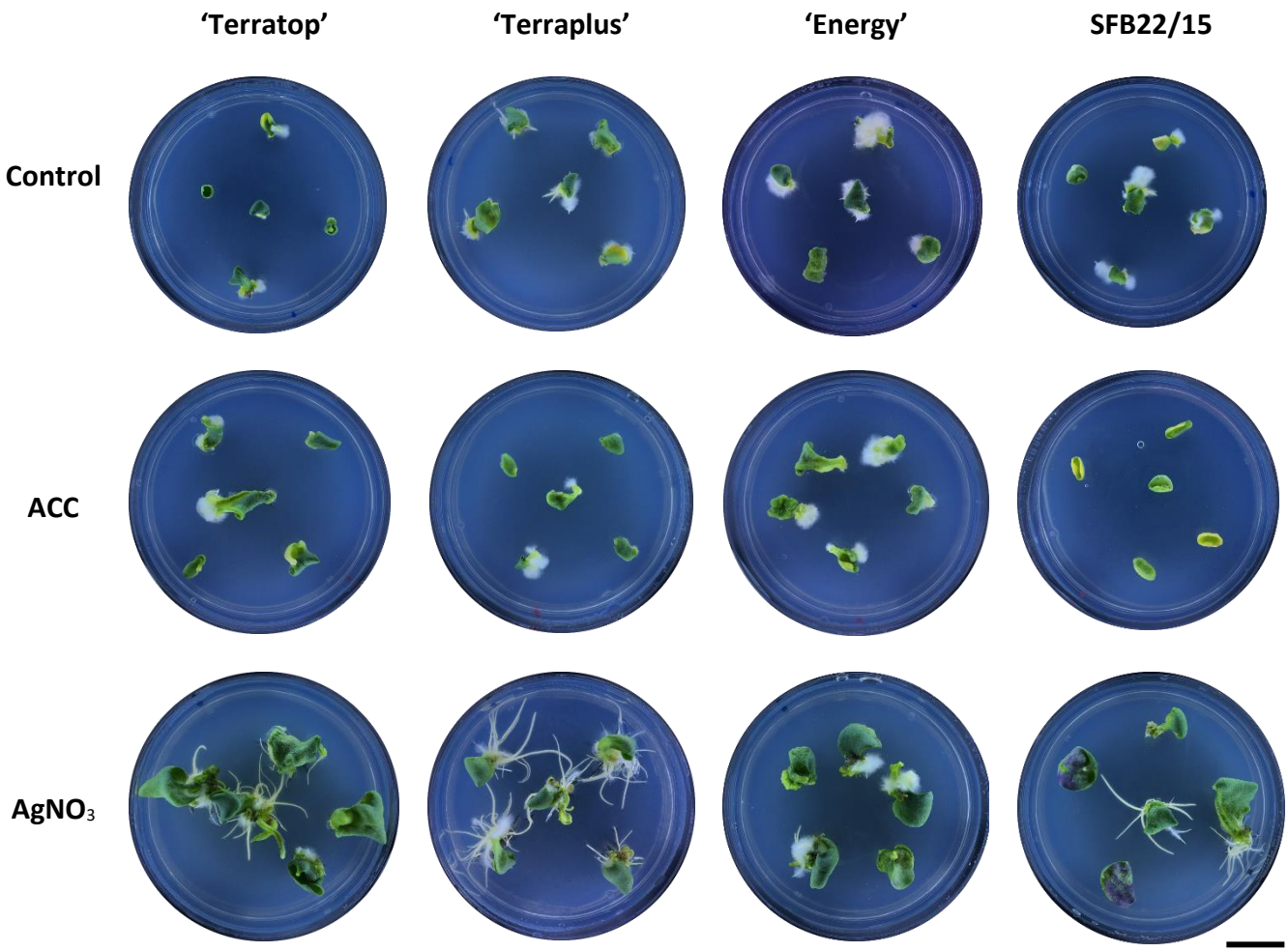
Supplemental Figure S1. Explant preparation from 5-days old seedlings of the European mustard line 'Terratop'. Bars= 1 cm



Supplemental Figure S2. Effect of plant growth regulators on adventitious shoot regeneration from hypocotyl explants of four lines of *Brassica juncea* (three European lines: 'Terratop', 'Terraplus', 'Energy 'and an Indian line CR2664). MS salts and vitamins were used in all media and explants were cultivated for 4 weeks on the different media SIM 2: 44.4 µM BAP + 0.49 µM IBA, SIM 3: 8.88 µM BAP + 0.49 µM IBA and SIM 4: 17.76 µM BAP + 0.49µM IBA). Bar = 1 cm



Supplemental Figure S3. Effect of plant growth regulators on adventitious shoot regeneration from cotyledon explants of four lines of *Brassica juncea* (three European lines: 'Terratop', 'Terraplus', 'Energy', and an Indian line CR2664). MS salts and vitamins were used in all media and explants were cultivated for 2 weeks on the different media SIM 2: 44.4 µM BAP + 0.49 µM IBA, SIM 3: 8.88 µM BAP + 0.49 µM IBA and SIM 4: 17.76 µM BAP + 0.49µM IBA). Bar = 1 cm



Supplemental Figure S4. Effect of the ethylene precursor ACC and the ethylene action blocker $AgNO_3$ on adventitious regeneration from cotyledon explants of four European lines of *Brassica juncea* ('Terratop', 'Terraplus', 'Energy' and SFB22/15). MS salts and vitamins were used in all media variants and explants were cultivated for 2 weeks on the different media. Control: 8.88 µM BAP + 5.37µM NAA, ACC: 8.88 µM BAP + 5.37µM NAA + 500 µM ACC, $AgNO_3$(SIM 5): 8.88 µM BAP + 5.37µM NAA + 9.95 µM $AgNO_3$.

Supplemental materials for chapter 3

Supplemental information: Antibody selection in microtiter plate

The antibody selection was performed as described previously with modifications (Russo *et al.*, 2018). In brief antibodies were selected using the naïve libraries HAL9 and HAL10 (Kügler *et al.*, 2015) on a biotinylated peptide (Monsalve *et al.*, 1993) (Peps4LS GmbH, Heidelberg, Germany) in microtiter plates (Corning, New York, USA). The peptide was ordered with two different linkers to prevent the enrichment of binders against the linker. For the panning procedure, 200ng/µL of Streptavidin diluted in PBS (137 mM NaCl; 1.76 mM KH_2PO_4 x 2 H_2O) was immobilized on a Costar Highbinding 96 well plate (Corning, New York, USA). Next, the wells were blocked with 350 µL 2 % BSA (2 % (w/v) bovine serum albumin in PBS; 0.05 % Tween20) for 1 hour at room temperature and then washed 3 times with PBST (PBS; 0.05 % Tween20). 1 µg of the peptide was diluted in PBS and coated in the wells at 4°C overnight and then washed 3 times with PBST. Before adding the libraries to the coated wells, the libraries ($5x10^{10}$ phage particles) were preincubated with 2% BSA on blocked wells for 1 hour at room temperature. Additionally, 5µg of streptavidin was added as competition to reduce the amount of streptavidin binders. The libraries were transferred to the coated wells, incubated for 2 hours at room temperature and washed 10 times. Bound phage was eluted with trypsin (10 µg/mL) at 37°C. The eluted phage was transferred to a 96 deep well plate (Greiner Bio-One, Frickenhausen, Germany) and incubated with 150 µL *E. coli* TG1 (OD_{600} = 0.5) firstly for 30 min at 37°C, then 30 min at 37°C and 650 rpm to infect the phage particles. 1 mL 2 x YT-GA (1.6% (w/v) Tryptone; 1% (w/v) Yeast extract; 0.5% (w/v) NaCl (pH 7.0), 100 mM D-Glucose, 100 µg/mL ampicillin) was added and incubated for 1 h at 37°C and 650 rpm, followed by addition of $1x10^{10}$ cfu M13KO7 helper phage. Subsequently, the infected bacteria were incubated for 30 min at 37°C followed by 30 min at 37°C and 650 rpm before centrifugation for 10 min at 3220 x *g*. The supernatant was discarded and the pellet resuspended in fresh 2 x YT-AK (1.6% (w/v) Tryptone; 1% (w/v) Yeast extract; 0.5% (w/v) NaCl (pH 7.0), 100 µg/mL ampicillin, 50 µg/mL kanamycin). The phage antibodies were amplified overnight at 30°C and

650 rpm and used for the next panning round. In total three panning rounds were performed. In each round, the stringency of the washing procedures was increased (20 x in panning round 2 and 30 x in panning round 3) and each round was alternated between the peptides with different linkers. After the third panning round, the titer was determined and the titer plate was used to select monoclonal antibody clones for the screening ELISA.

Production of soluble antibodies in MTPs and screening ELISA

Soluble antibody fragments (scFv) were produced in 96-well MTPs with polypropylene (U96 PP, Greiner Bio-One). Briefly, 150 µL 2 x YT-GA was inoculated with the bacteria bearing scFv expressing phagemids. MTPs were incubated overnight at 37°C and 850 rpm in a MTP shaker (Thermoshaker PST-60HL-4, Lab4You, Berlin, Germany). A volume of 180 µL 2 x YT-GA in a PP-MTP well was inoculated with 10 µL of the overnight culture and grown at 37°C and 850 rpm until bacteria reached an OD_{600} of 0.5. Bacteria were harvested by centrifugation for 10 min at 3,220 x *g* and the supernatant was discarded. To induce expression of the antibody genes, the pellets were resuspended in 180 µL 2 x YT supplemented with 100 µg/mL ampicillin and 50 µM isopropyl-beta D thiogalacto pyranoside (IPTG) and incubated at 30°C and 850 *rpm* overnight. Bacteria were pelleted by centrifugation for 10 min at 3,220 x *g* and 4°C. The scFv-containing supernatant was transferred to a new PP-MTP and stored at 4°C before ELISA analysis.

For the ELISA, 200 ng/µL of Streptavidin was coated on 96 well microtiter plates Costar Highbinding 96 well plate (Corning, New York, USA) in PBS (pH 7.4) for 1 hour at room temperature. After coating wells are blocked with 2% BSA in PBST for 1 hour at room temperature, followed by three washing steps with PBST. 100ng/µL of peptide were incubated overnight at 4°C. Supernatants containing monoclonal scFv were mixed with 2% BSA in PBST (1:2) and incubated in the antigen coated plates for 1.5 hours at room temperature followed by three PBST washing cycles. Bound scFv were detected using murine mAb 9E10 which recognizes the C-terminal c-myc tag (1:50 diluted in 2% BSA in PBST) and a goat anti-mouse

serum conjugated with horseradish peroxidase (HRP) (A0168, Sigma) (1:42,000 dilution in 2% BSA in PBST). Bound antibodies were visualized with tetramethylbenzidine (TMB) substrate (20 parts TMB solution A (30 mM Potassium citrate; 1% (w/v) Citric acid (pH 4.1)) and 1 part TMB solution B (10 mM TMB; 10% (v/v) Acetone; 90% (v/v) Ethanol; 80 mM H_2O_2 (30%)) were mixed). After stopping the reaction by addition of 1 N H_2SO_4, absorbance at 450 nm with a 620 nm reference was measured in an ELISA plate reader (Epoch, BioTek, Bad Friedrichshall, Germany). Monoclonal binders were sequenced and analyzed using VBASE2 (www.vbase2.org) (Mollova *et al.*, 2010).

scFv-Fc cloning, expression and purification

The cloning, expression and purification was performed as previously (Jäger *et al.*, 2013) described. In brief the unique scFv sequences isolated by antibody-phage display in MTPs were subcloned into pCSE2.6-hIgG1-Fc-XP using NcoI/NotI (New England Biolabs, Frankfurt, Germany) for mammalian production as scFv-Fc, an IgG-like antibody format. EXPI293F (Thermo Fisher Scientific) cells were transfected. For production, the transfected EXPI293F cells were cultured in chemically defined medium F17 (Thermo Fisher Scientific) supplemented with 0.1% pluronic F68 (PAN-Biotech, Aidenbach, Germany) and 7.5 mM L-glutamine (Merck). A subsequent protein A purification was performed as described previously.

Antibody titration ELISA

The titration ELISA was performed similar to the indirect antigen ELISA (see above). A dilution series was made in blocking solution starting with 1 µg antibody. Afterwards, 100 µL of each dilution was added per well and incubated for 1 hour at room temperature. The plate was washed 3 times with PBST. Goat α-human IgG (Fc-specific, A0170, Sigma) conjugated to horse radish peroxidase (HRP, 1:70,000) was used as the secondary detection antibody and was incubated for 1 hour at room temperature. Unbound antibodies were removed by additional washing steps. Bound antibodies were visualized with TMB substrate (20 parts TMB

solution A and 1 part TMB solution B). After stopping the reaction by adding 100 µL 1N H_2SO_4, absorbance at 450 nm with a 620 nm reference was measured in an ELISA plate reader.

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Supplemental Table S1. Transformation efficiencies for two *Brassica juncea* lines (Terratop and CR2664) in different experiments using the three vectors pEGFP, pBraj1256 and pBraj3477**.**

Experiment No.	Vector	Line	No. of explants[†]	No. of T_0 lines obtained	Transformation efficiency (%)[‡]	Transgenic lines (T_0)[§]
1	pEGFP	Terratop	100	0	0	-
		CR2664	100	5	5	-
2	pEGFP	Terratop	100	1	1	-
		CR2664	100	4	4	-
3	pEGFP	Terratop	100	1	1	-
		CR2664	100	2	2	-
4	pBraj1256	CR2664	300	10	3.5	1;2;3;4;5;6;7;8;17;18
	pBraj3477	CR2664	300	9	3	9;10;11;12;13;14;15;19;20
5	pBraj1256	Terratop	100	0	0	0
	pBraj3477	Terratop	100	2	2	16;21
6	pBraj1256	CR2664	100	6	6	24;25;27;28;29;30
	pBraj3477	CR2664	100	3	3	22;23;26
7	pBraj1256	Terratop	100	1	1	31
	pBraj3477	Terratop	100	0	0	0
8	pBraj1256	CR2664	100	3	3	35;36;37
	pBraj3477	CR2664	100	3	3	32;33;34
9	pBraj1256	Terratop	200	7	3.5	47;48;49;50;51; 52;53
	pBraj3477	Terratop	200	13	6.5	38,39;40;41;42;43;44;45;46;54;55;56;57

[†]: See these data in condensed form in Table 1.

[‡]: Percentage of independent transgenic shoots per 100 explants. Transgenicity was analyzed by three different PCR reactions targeting the T-DNA.

[§]: Designation of T_0 lines.

Supplemental Table S2. Genetic characterization of T_0 plants regarding transgene presence, seed weight and germination of seeds of the *Brassica juncea* lines Terratop and CR2664.

Plant number	Plasmid	Line	Transgenic assay[†]	Genotype[‡]		Seed number obtained from free pollination	Weight of 100 seeds [g] [§]	In vitro germination [%] (n = 10)	Ex vitro germination [%] (n = 20)
				Bra j IA allele 1 / allele 2	***Bra j IB*** allele 1 / allele 2				
21	pBraj3477	Terratop	+	n.a.	- 566 bp / n.a.	n.a.	n.a.	n.a.	n.a.
24	pBraj1256	CR2664	+	+ 1 bp (sg1) / - 1 bp (sg1)	+ 2 bp (sg1) / - 2 bp (sg1)	115	0.310	50	0
28	pBraj1256	CR2664	+	+ 1 bp (sg1), + 1 bp (sg6) / - 35 bp (sg1)	- 58 bp (sg1, sg2) / - 7 bp (sg1)	886	0.347*	70	5
29	pBraj1256	CR2664	+	+ 1 bp (sg1) / - 1 bp (sg1)	+ 2 bp (sg1) / - 2 bp, 1SNP (sg2)	260	0.279	0	0
35	pBraj1256	CR2664	+	+ 1 bp (sg1), - 1 bp (sg5) / + 1 bp (sg1), - 1 bp (sg5)	- 32 bp (sg1, sg2), - 83 bp (sg5, sg6) / - 48 bp, 1 SNP (sg1, sg2)	91	0.324	100	70
36	pBraj1256	CR2664	+	+1 bp (sg1) / - 3 bp (sg1)	- 4 bp (sg1) / wild type	110	0.097	30	60

39	pBraj3477	Terratop	+	wild type / wild type	wild type / wild type	n.a.	n.a.	n.a.	n.a.
40	pBraj3477	Terratop	+	wild type / wild type	wild type / wild type	n.a.	n.a.	n.a.	n.a.
41	pBraj3477	Terratop	+	wild type / wild type	wild type / wild type	n.a.	n.a.	n.a.	n.a.
42	pBraj3477	Terratop	+	+ 1 bp (sg 4) / wild type	- 1 bp (sg7) / - 1 bp (sg7)	n.a.	n.a.	n.a.	n.a.
43	pBraj3477	Terratop	+	wild type / wild type	wild type / wild type	n.a.	n.a.	n.a.	n.a.
45	pBraj3477	Terratop	+	wild type / wild type	wild type / wild type	n.a.	n.a.	n.a.	n.a.
46	pBraj3477	Terratop	+	wild type / wild type	wild type / wild type	n.a.	n.a.	n.a.	n.a.
47	pBraj1256	Terratop	+	- 4 bp (sg5) / wild type	- 790 bp / -32 bp (sg1, sg2)	n.a.	n.a.	n.a.	n.a.
50	pBraj1256	Terratop	+	-1 bp (sg 1) / -3 bp (sg1)	+ 14 bp (sg 1) / wild type	n.a.	n.a.	n.a.	n.a.
51	pBraj1256	Terratop	+	indels not clear	indels not clear	n.a.	n.a.	n.a.	n.a.

				(sg1, sg5)	(sg1)				
52	pBraj1256	Terratop	+	indels not clear (sg1, sg5)	indels not clear (sg1)	n.a.	n.a.	n.a.	n.a.
53	pBraj1256	Terratop	+	- 1 bp (sg1) / - 3 bp (sg1)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
54	pBraj3477	Terratop	+	wild type / wild type	wild type / wild type	n.a.	n.a.	n.a.	n.a.
55	pBraj3477	Terratop	+	+ 1 bp (sg7) / - 109 bp (sg4, sg7)	- 629 bp (sg 7)/ wild type	n.a.	n.a.	n.a.	n.a.
56	pBraj3477	Terratop	+	wild type / wild type	wild type / wild type	n.a.	n.a.	n.a.	n.a.
57	pBraj3477	Terratop	+	wild type / wild type	+ 1 bp (sg7) / + 1 bp (sg7)	n.a.	n.a.	n.a.	n.a.

†: PCR with three T-DNA-specific primer pairs. “+“ fragment detected, “- “ no fragment detected.

‡: The sequencing chromatograms were decoded by ICE Analysis (https://ice.synthego.com/#/).

§: for lines with less than 300 seeds no statistical analysis was done. For the remaining lines the 100 seed weight of 3-4 subsamples was compared against the wild type by Dunnett’s test, * = $p < 0.05$; ns = not significant

n.a.: not analyzed.

Supplemental Table S3. Genetic characterization of T_1 plants regarding transgene presence, seed weight and germination of seeds of the *Brassica juncea* lines Terratop and CR2664.

Plant number	Plasmid	Line	Transgenic assay[†]	Genotype[‡]		Seed number obtained from free pollination	Weight of 100 seeds [g] [§]	In vitro germination [%] (n = 10)	Ex vitro germination [%] (n = 20)
				Bra j IA allele 1 / allele 2	***Bra j IB*** allele 1 / allele 2				
1-1	pBraj1256	CR2664	+	- 1 bp (sg1) / - 1 bp (sg1)	+ 1 bp (sg1) / + 1 bp (sg1)	n.a.	n.a.	n.a.	n.a.
1-2	pBraj1256	CR2664	-	- 1 bp (sg1) / - 1 bp (sg1)	+ 1 bp (sg1) / + 1 bp (sg1)	n.a.	n.a.	n.a.	n.a.
23-1	pBraj3477	CR2664	+	+ 1 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-2	pBraj3477	CR2664	+	- 14 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-3	pBraj3477	CR2664	+	- 14 bp (sg 7) / - 14 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-4	pBraj3477	CR2664	+	+ 1 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-5	pBraj3477	CR2664	+	- 14 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-6	pBraj3477	CR2664	+	+ 1 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-7	pBraj3477	CR2664	+	- 14 bp (sg 7) / - 14 bp (sg 7)	+ 1 bp (sg 7) / wild type	n.a.	n.a.	n.a.	n.a.
23-8	pBraj3477	CR2664	+	- 14 bp (sg 7) / - 14 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.

23-9	pBraj3477	CR2664	+	- 14 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-10	pBraj3477	CR2664	+	- 14 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-11	pBraj3477	CR2664	+	+ 1 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-12	pBraj3477	CR2664	+	+ 1 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-13	pBraj3477	CR2664	+	+ 1 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-14	pBraj3477	CR2664	+	+ 1 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-15	pBraj3477	CR2664	+	- 14 bp (sg 7) / + 1 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-16	pBraj3477	CR2664	+	- 14 bp (sg 7) / - 14 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
23-17	pBraj3477	CR2664	+	- 14 bp (sg 7) / - 14 bp (sg 7)	wild type / wild type	n.a.	n.a.	n.a.	n.a.
31-9	pBrj1256	Terratop	+	n.a.	n.a.	1256	0.330***	100	90
31-12	pBrj1256	Terratop	+	n.a.	n.a.	648	0.326***	30	75
35-2	pBrj1256	CR2664	+	n.a.	n.a.	0	n.a.	n.a.	n.a.
35-6	pBrj1256	CR2664	+	n.a.	n.a.	23	0.124	n.a.	n.a.

35-9	pBrj1256	CR2664	+	n.a.	n.a.	2031	0.151***	100	95
35-11	pBrj1256	CR2664	+	n.a.	n.a.	742	0.196***	100	100
35-13	pBrj1256	CR2664	+	n.a.	n.a.	2085	0.253^{ns}	100	95

†: PCR with three T-DNA-specific primer pairs. “+“ fragment detected, “- “ no fragment detected.

‡: The sequencing chromatograms were decoded by ICE Analysis (https://ice.synthego.com/#/).

§: for lines with less than 300 seeds no statistical analysis was done. For the remaining lines the 100 seed weight of 3-4 subsamples was compared against the wild type by Dunnett’s test, *** = $p < 0.0001$; ns = not significant

n.a.: not analyzed.

Supplemental Table S4. Controlled pollinated flowers of T_0 plants of *Brassica juncea* lines Terratop and CR2664 with the number of seeds and seed weight.

Plant number	Plasmid	Line	Number of controlled pollinated flowers	No of siliques formed (%)	Seed number obtained from controlled pollination	Seed number per silique	Weight of 100 seeds
K2	-	Terratop	20	18 (90%)	306	17.0	0.233
K17	-	CR2664	20	18 (90%)	254	14.1	0.275
1	pBraj1256	CR2664	20	18 (90%)	188	10.4	0.333
22	pBra3477	CR2664	16	14 (70%)	45	3.2	0.346
23	pBra3477	CR2664	20	18 (90%)	84	4.7	0.213
24	pBraj1256	CR2664	10	7 (70%)	16	2.3	0.281
25	pBra1256	CR2664	18	11 (61 %)	26	2.4	0.112
26	pBra3477	CR2664	15	15 (100%)	115	7.7	0.112
27	pBraj1256	CR2664	15	10 (67%)	40	4.0	0.07
28	pBraj1256	CR2664	20	20 (100 %)	166	8.3	0.318
29	pBraj1256	CR2664	10	10 (100 %)	83	8.3	0.342
30	pBraj1256	CR2664	20	9 (45%)	56	6.2	0.165
31	pBraj1256	Terratop	20	17 (85%)	104	6.1	0.200
32	pBra3477	CR2664	20	15 (75%)	44	2.9	0.238
33	pBra3477	CR2664	16	16 (100%)	172	10.8	0.216
34	pBra3477	CR2664	20	19 (95%)	64	3.4	0.292
35	pBraj1256	CR2664	10	7 (70%)	12	1.7	0.216
36	pBraj1256	CR2664	11	9 (82%)	53	5.9	0.213
37	pBraj1256	CR2664	20	16 (80%)	63	3.9	0.227

Supplemental Table S5. MoClo compatible vectors used in this study.

Name	MoClo level	Application	
pDI1	level 0 accepter	sgRNA expression cassette accepter in position 1	Generated in this work
pDI2	level 0 accepter	sgRNA expression cassette accepter in position 2	Generated in this work
pDI3	level 0 accepter	sgRNA expression cassette accepter in position 3	Generated in this work
pDI4E	level 0 accepter	sgRNA expression cassette accepter in position 4	Generated in this work
pDI1-sg1	level 0	*AtU6-26::sg1* expression cassette in position 1	Generated in this work
pDI1-sg3	level 0	*AtU6-26::sg3* expression cassette in position 1	Generated in this work
pDI2-sg2	level 0	*AtU6-26::sg2* expression cassette in position 2	Generated in this work
pDI2-sg4	level 0	*AtU6-26::sg4* expression cassette in position 2	Generated in this work
pDI3-sg5	level 0	*AtU6-26::sg5* expression cassette in position 3	Generated in this work
pDI3-sg7A	level 0	*AtU6-26::sg7A* expression cassette in position 3	Generated in this work
pDI4E-sg6	level 0	*AtU6-26::sg6* expression cassette in position 4	Generated in this work
pDI4E-sg7B	level 0	*AtU6-26::sg7B* expression cassette in position 4	Generated in this work
pICH47732	level 1 accepter	MoClo level 1 accepter in position 1	Weber et al., 2011
pICH47742	level 1 accepter	MoClo level 1 accepter in position 2	Weber et al., 2011
pICH47752	level 1 accepter	MoClo level 1 accepter in position 3	Weber et al., 2011
pMC181	level 1	*2x35S::nptII* expression cassette in position 1	Weber et al., 2011
pMC170	level 1	*2x35S::HPT* expression cassette in position 1	Generated in this work
pEGFP1	level 1	2x*35S*::*EGFP* expression cassette in position 1	Generated in this work
pMC63	level 1	*2x35S::SpCas9* expression cassette in position 2	Generated in this work
psg1256_P3	level 1	four sgRNA (sg1, sg2, sg5, sg6) expression cassettes in position 3	Generated in this work
psg3477_P3	level 1	four sgRNA (sg3, sg4, sg7A, sg7B) expression cassettes in position 3	Generated in this work
pICH50881	End-linker 2	end-linker 2 for level M cloning	Weber et al., 2011
pICH50892	End-linker 3	end-linker 3 for level M cloning	Weber et al., 2011
pAGM8031	level M accepter	Final constract accepter	Weber et al., 2011
pEGFP	level M	Final constract	Generated in this work
pBraj1256	level M	Final constract	Generated in this work
pBraj3477	level M	Final constract	Generated in this work

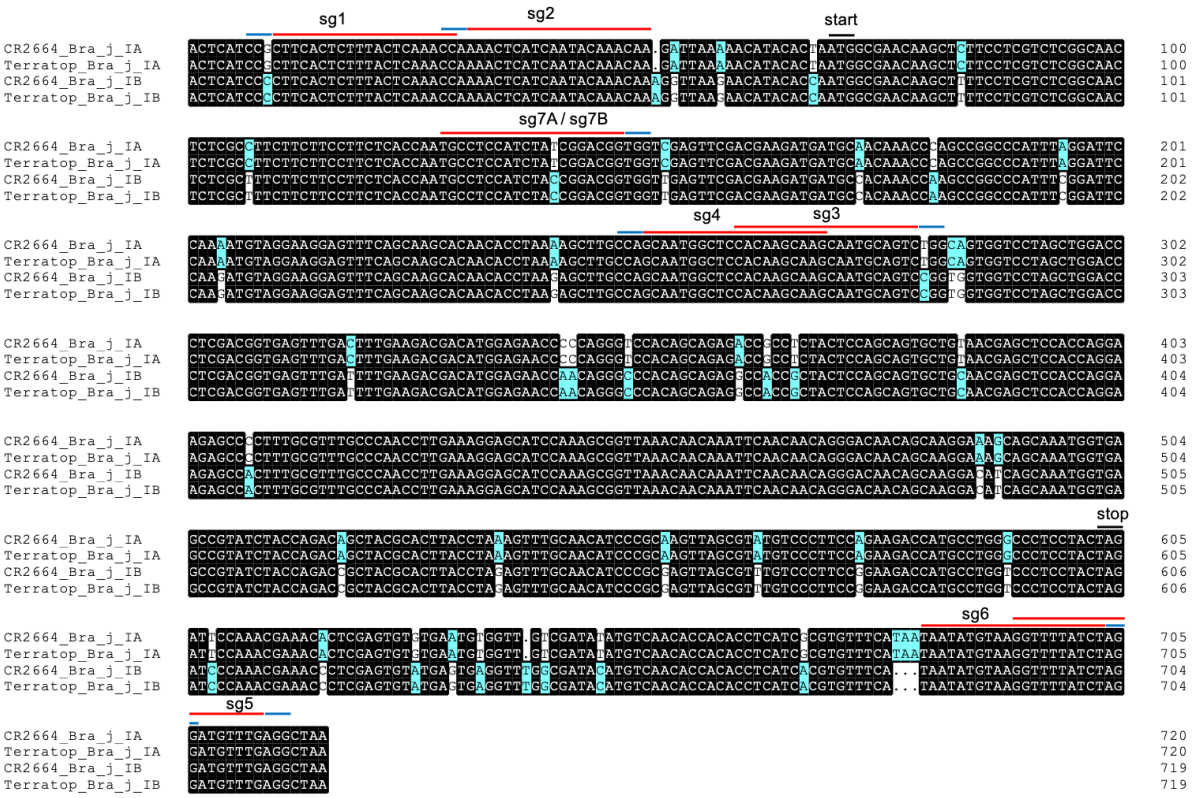
Supplemental Table S6. sgRNA sequences. Eight sgRNAs were designed by CRISPR-P (http://cbi.hzau.edu.cn/ CRISPR2/). On-score: predicted on-target efficiency score of sgRNA. PAMs are in bold. sgRNAs editing efficiency of both mustard lines were calculated from Table 2 and Table S2.

Name	Sequence (5'-3')	GC (%)	On-score	Editing efficiency (%)	
				CR2664	Terratop
sg1	GGTTTGAGTAAAGAGTGAAG**CGG**	40	0.1933	80(8/10)	100(6/6)
sg2	TTGTTTGTATTGATGAGTTT**TGG**	25	0.2178	40(4/10)	16.7(1/6)
sg3	CACAAGCAAGCAATGCAGTC**TGG**	50	0.0372	0(0/6)	8.3(1/12)
sg4	CTTGCTTGTGGAGCCATTGC**TGG**	55	0.1477	33.3(2/6)	25(3/12)
sg5	GGTTTTATCTAGGATGTTTG**AGG**	35	0.1864	20(2/10)	50(3/6)
sg6	TAATATGTAAGGTTTTATCT**AGG**	20	0.1933	30(3/10)	0(0/6)
sg7A	TGCCTCCATCTATCGGACGG**TGG**	60	0.1933	100(6/6)	8.3(1/12)
sg7B	TGCCTCCATCTACCGGACGG**TGG**	65	0.1933	100(6/6)	25(3/12)

Supplemental Table S7. Oligonucleotides used in this study.

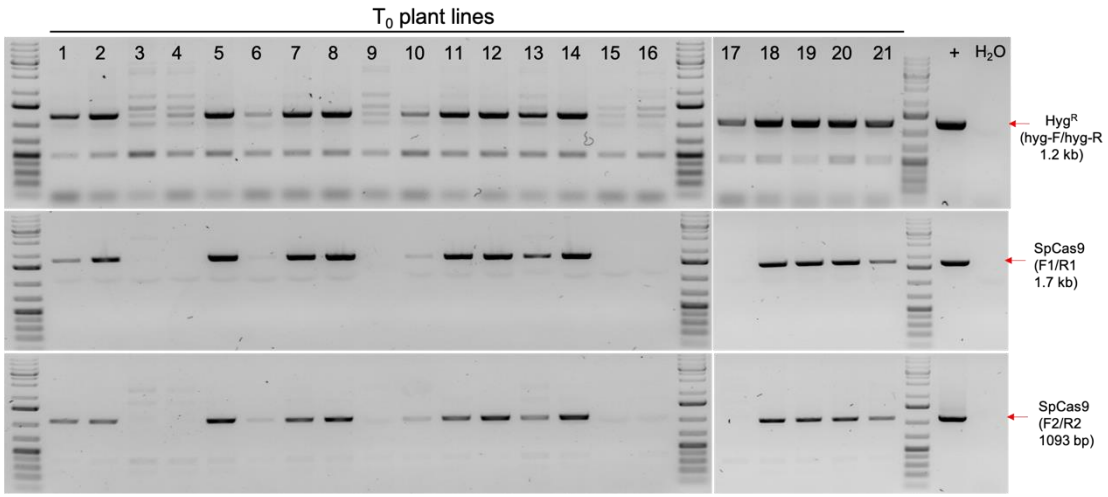
Name	Sequence (5'-3')	Application
BraJI-sg1-F	ATTGGTTTGAGTAAAGAGTGAAG	Construction of sgRNA (sg1)
BraJI-sg1-R	AAACCTTCACTCTTTACTCAAAC	
BraJI-sg2-F	ATTGTTGTTTGTATTGATGAGTTT	Construction of sgRNA (sg2)
BraJI-sg2-R	AAACAAACTCATCAATACAAACAA	
BraJI-sg3-F	ATTGCACAAGCAAGCAATGCAGTC	Construction of sgRNA (sg3)
BraJI-sg3-R	AAACGACTGCATTGCTTGCTTGTG	
BraJI-sg4-F	ATTGCTTGCTTGTGGAGCCATTGC	Construction of sgRNA (sg4)
BraJI-sg4-R	AAACGCAATGGCTCCACAAGCAAG	
BraJI-sg5-F	ATTGGTTTTATCTAGGATGTTTG	Construction of sgRNA (sg5)
BraJI-sg5-R	AAACCAAACATCCTAGATAAAAC	
BraJI-sg6-F	ATTGTAATATGTAAGGTTTTATCT	Construction of sgRNA (sg6)
BraJI-sg6-R	AAACAGATAAAACCTTACATATTA	
BraJI-sg7A-F	ATTGTGCCTCCATCTATCGGACGG	Construction of sgRNA (sg7A)
BraJI-sg7A-R	AAACCCGTCCGATAGATGGAGGCA	
BraJI-sg7B-F	ATTGTGCCTCCATCTACCGGACGG	Construction of sgRNA (sg7B)
BraJI-sg7B-R	AAACCCGTCCGGTAGATGGAGGCA	
BrajA-F	GAAACAAAGGGACAATGACG	Amplification of *Bra j IA* site
BrajA-R	AAACCGTTCGGCTCCTATC	
BrajB-F	CACCAGTTGTTTCTCCACG	Amplification of *Bra j IB* site
BrajB-R	TTAGACTCCTATCCACTTCGC	
hyg-F	ATGAAAAAGCCTGAACTCACCGC	Detection of Hygromycin
hyg-R	CTATTCCTTTGCCCTCGGACG	
F1	GAACTCGTCAAAGTAATGGG	Detection of Cas9
R1	CGCCCAAGTTGGTCAGAGTA	
F2	CGATATCGGCACAAACAGCG	Detection of Cas9
R2	GTAAAATTCCTCCTGGCTTG	
NptII-F	ATGGTTGAACAAGATGGATT	Amplification of Kanamycin
NptII-R	TCAGAAGAACTCGTCAAGAA	
BrajI-sg1-OT1-F	GACAGCGTTTGGTGGTAGGT	Amplification of sg1 off-target site 1 (sg1-OT1)
BrajI-sg1-OT1-R	CGGACTAAGAGCGTGTGACC	
BrajI-sg1-OT2-F	GCTGCCTCTTCAAACTCTGGA	Amplification of sg1 off-target site 2 (sg1-OT2)
BrajI-sg1-OT2-R	TGAACTCTTTGGTGGGCGTT	
BrajI-sg1-OT3-F	AAGAGGTGGCTCCACTGAGA	Amplification of sg1 off-target site 3 (sg1-OT3)
BrajI-sg1-OT3-R	TATCGGACGAAAGTCGAGATCC	
BrajI-sg2-OT1-F	AGCCTCAGTCTTCGGAGTCT	Amplification of sg2 off-target site 1 (sg2-OT1)
BrajI-sg2-OT1-R	GACGAACGACTAACTTGCGC	
BrajI-sg2-OT2-F	TGCAGATGCGGATGACTGTT	Amplification of sg2 off-target site 2 (sg2-OT2)
BrajI-sg2-OT2-R	CACACTCTTCGCCGTGGTAT	
BrajI-sg2-OT3-F	ACGATGGTACCCACTACCCA	

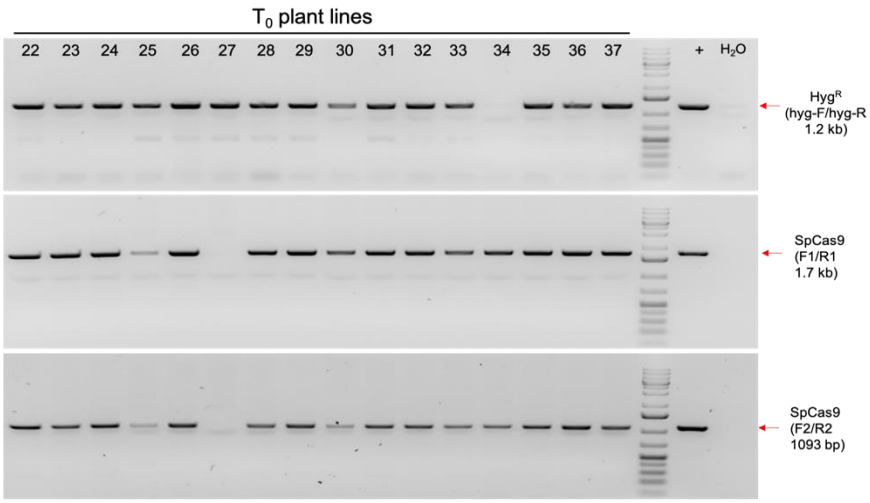
BrajI-sg2-OT3-R	GCCAGGTATGAGGACTCGTG	Amplification of sg2 off-target site 3 (sg2-OT3)
BrajI-sg3-OT1-F	CCTCCAAGTCCCGTTTCGAA	Amplification of sg3 off-target site 1 (sg3-OT1)
BrajI-sg3-OT1-R	CTGTGCTTCGCCATCAATCG	
BrajI-sg3-OT2-F	TCCCTGCATTTGTTGTCCCT	Amplification of sg3 off-target site 2 (sg3-OT2)
BrajI-sg3-OT2-R	TTTACACGTGATCGCCATGC	
BrajI-sg3-OT3-F	TGCAGATGCGGATGACTGTT	Amplification of sg3 off-target site 3 (sg3-OT3)
BrajI-sg3-OT3-R	CACACTCTTCGCCGTGGTAT	
BrajI-sg4-OT1-F	ATGCTAACTCGCGGGATGTT	Amplification of sg4 off-target site 1 (sg4-OT1)
BrajI-sg4-OT1-R	CATGATGGCGTGACATGAGC	
BrajI-sg4-OT2-F	GGCATGGTCTTCTGGAAGGG	Amplification of sg4 off-target site 2 (sg4-OT2)
BrajI-sg4-OT2-R	ACCAACACTCCACACTTCCC	
BrajI-sg4-OT3-F	CAGGAAGGACCAAGAGCGTT	Amplification of sg4 off-target site 3 (sg4-OT3)
BrajI-sg4-OT3-R	TCAGTTTGGTTCGGCAGGTC	
BrajI-sg5-OT1-F	TCTTCCTTCTCACCAACGCC	Amplification of sg5 off-target site 1 (sg5-OT1)
BrajI-sg5-OT1-R	TCACGTAGATTCGCCCATGG	
BrajI-sg5-OT2-F	GCAACTGCCCTTAGTCCACA	Amplification of sg5 off-target site 2 (sg5-OT2)
BrajI-sg5-OT2-R	TCTTGGAGTGATGTCGCAGG	
BrajI-sg5-OT3-F	TCGTTGCAGGTGAAGTTCCT	Amplification of sg5 off-target site 3 (sg5-OT3)
BrajI-sg5-OT3-R	CGAGGAACACACACCTGAGA	
BrajI-sg6-OT1-F	AGGTGATAGCTGACCTTGCG	Amplification of sg6 off-target site 1 (sg6-OT1)
BrajI-sg6-OT1-R	AAGACGAGCAGTTACCACCG	
BrajI-sg6-OT2-F	TTCCATGCGTCTGCTTCCTT	Amplification of sg6 off-target site 2 (sg6-OT2)
BrajI-sg6-OT2-R	ACCATTGACGCAGCGGATAT	
BrajI-sg6-OT3-F	CGAGATCCAAGCACAGTGGT	Amplification of sg6 off-target site 3 (sg6-OT3)
BrajI-sg6-OT3-R	AGAGTACATGCAACGCCCAT	
BrajI-sg7A-OT1-F	TTCTTGTTGCAGGGAGCCAT	Amplification of sg7A off-target site 1 (sg7A-OT1)
BrajI-sg7A-OT1-R	ATCACCAAGATGCCGTTCGT	
BrajI-sg7A-OT2-F	TGCATGTGGTTTTCGGTTTCT	Amplification of sg7A off-target site 2 (sg7A-OT2)
BrajI-sg7A-OT2-R	TACTCGGCTCCTCCACTGTT	
BrajI-sg7A-OT3-F	CCATGGTGGGTCTTTCGGAT	Amplification of sg7A off-target site 3 (sg7A-OT3), and sg7B off-target site 2 (sg7B-OT2)
BrajI-sg7A-OT3-R	TGCCAGACACATGAAGCTGT	
BrajI-sg7B-OT1-F	AGGCTGCCACTGAATGTACA	Amplification of sg7B off-target site 1 (sg7B-OT1)
BrajI-sg7B-OT1-R	ACTTCGACTTCACCTACGCG	
BrajI-sg7B-OT3-F	GTTGCGTTCAAACTCAGCCG	Amplification of sg7B off-target site 3 (sg7B-OT3)
BrajI-sg7B-OT3-R	TGCGGGTAAAGGTTCATTGGA	

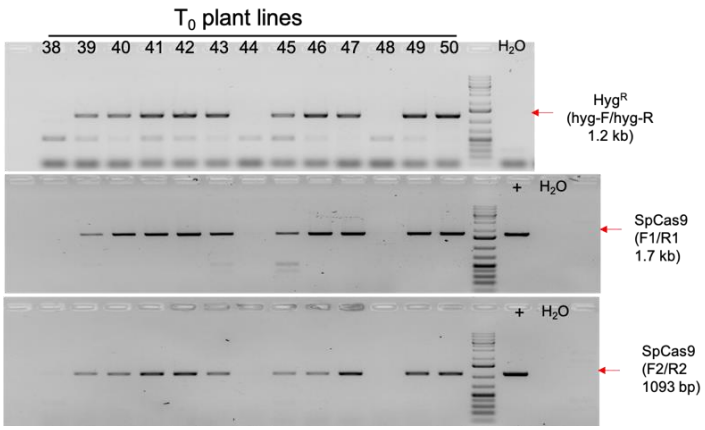


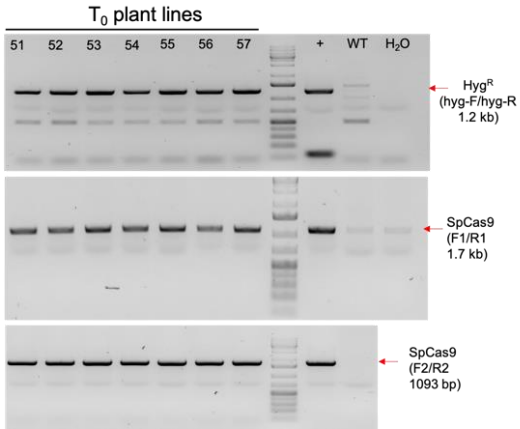
Supplemental Figure S1. Sequence alignment of *Bra j I* homoeologs in *B. juncea* varieties Terratop and CR2664. Target genomic regions were amplified by PCR using homoeolog-specific primers, subcloned and sequenced. sgRNAs-targeting sites are indicated by red lines, PAMs are indicated by blue lines, start codon (ATG) and stop codon (TAG) are indicated by black lines. The genes contain no introns.

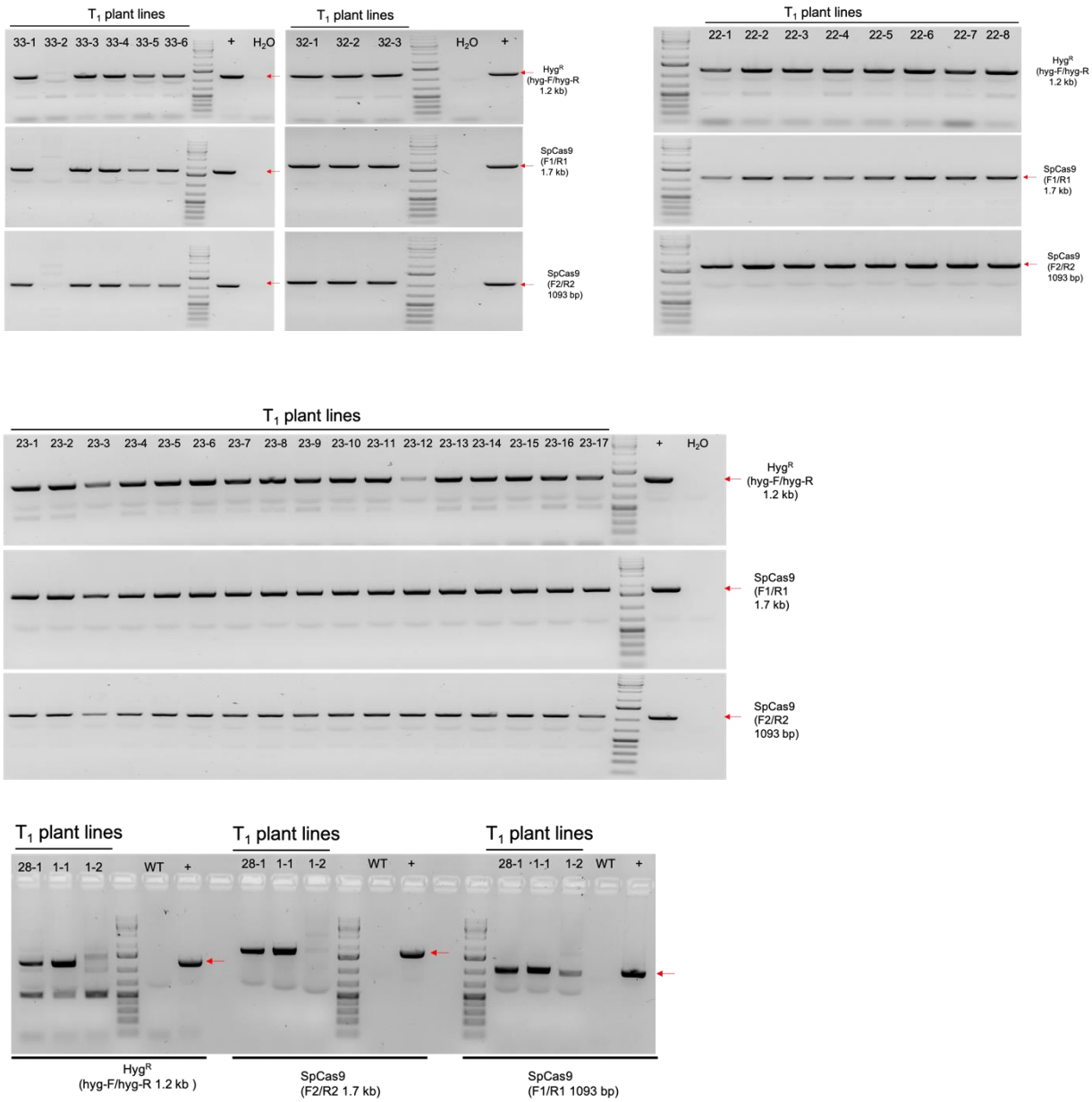

Hyg-F
Hyg-R
F1
R1
LB
p35S
Hyg^R
Ter
p35S
SpCas9
Ter
RB
F2
R2


T0 plant lines
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 + H2O
Hyg^R (hyg-F/hyg-R 1.2 kb)
SpCas9 (F1/R1 1.7 kb)
SpCas9 (F2/R2 1093 bp)

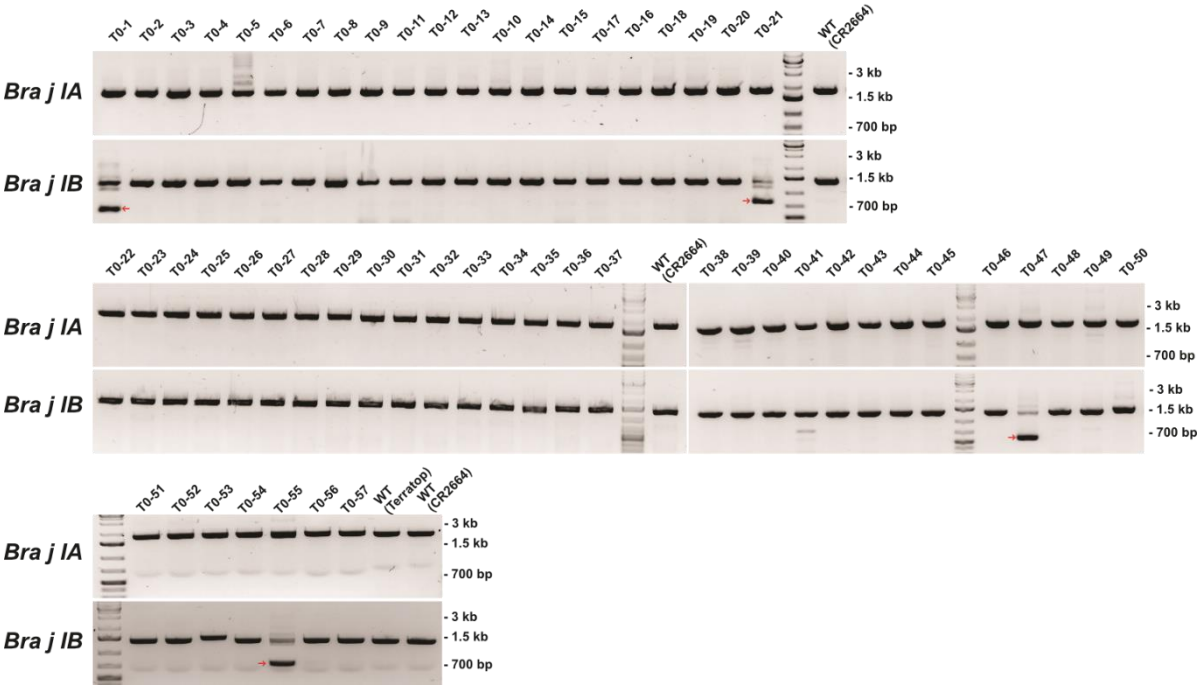

T0 plant lines
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 + H2O
Hyg^R (hyg-F/hyg-R 1.2 kb)
SpCas9 (F1/R1 1.7 kb)
SpCas9 (F2/R2 1093 bp)


T0 plant lines
38 39 40 41 42 43 44 45 46 47 48 49 50 H2O
Hyg^R (hyg-F/hyg-R 1.2 kb)
+ H2O
SpCas9 (F1/R1 1.7 kb)
+ H2O
SpCas9 (F2/R2 1093 bp)

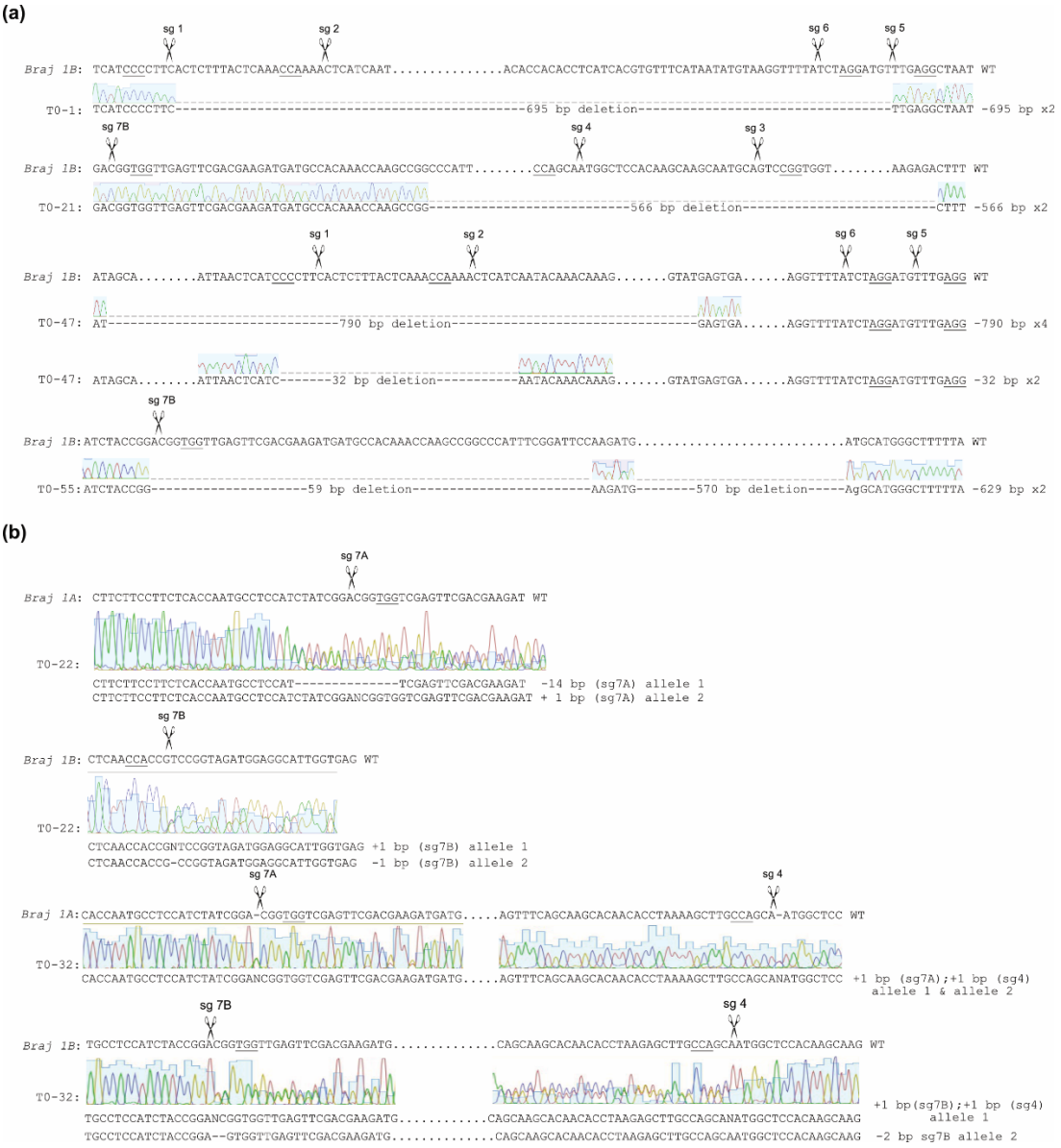

T0 plant lines
51 52 53 54 55 56 57 + WT H2O
Hyg^R (hyg-F/hyg-R 1.2 kb)
SpCas9 (F1/R1 1.7 kb)
SpCas9 (F2/R2 1093 bp)



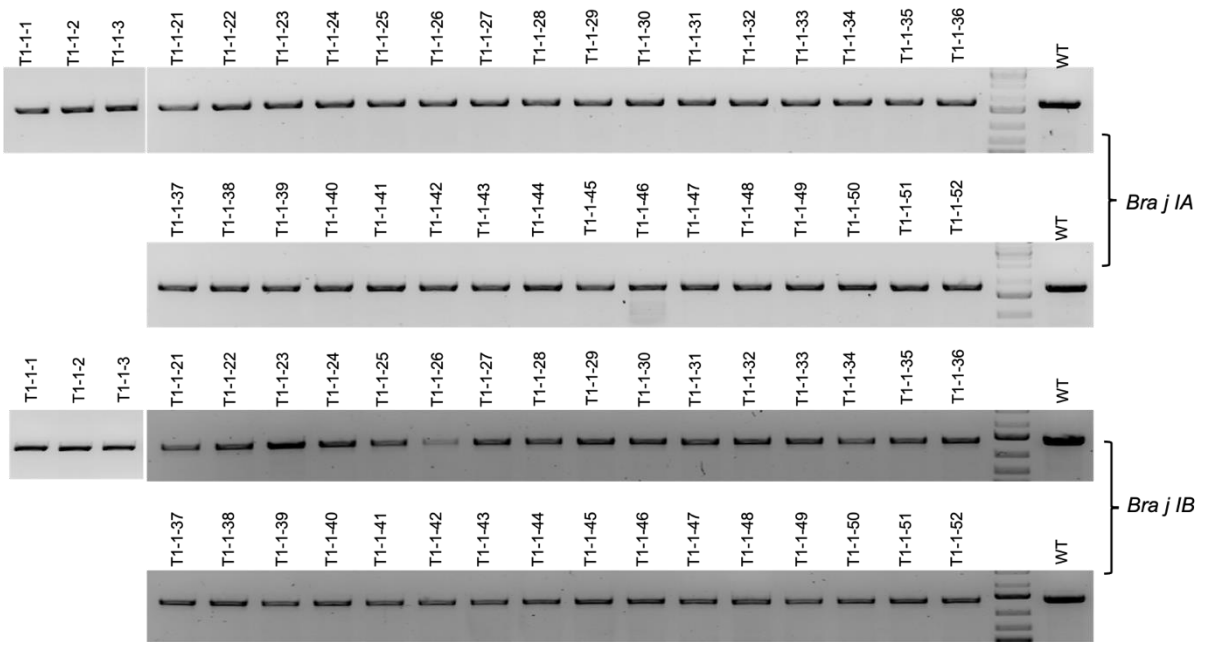
Supplemental Figure S2. Outcomes of the PCR test for transgenicity of all obtained mustard lines. 57 T_0 plants and 37 T_1 plants were analyzed by three different PCR reactions targeting the T-DNA, target fragments are indicated by red arrows. WT: wild type CR2664, +: plasmid control, H_20: water control.



Supplemental Figure S3. PCR amplification of both *Bra j I* homoeologs from genomic DNA of the T_0 lines. Homoeolog-specific primers were used to amplify *Bra j IA* and *Bra j IB* independently from the genome of edited mustard plants. Large deletions indicated by short fragment sizes (red arrows) were found in lines T0-1, T0-21, T0-47, and T0-55.

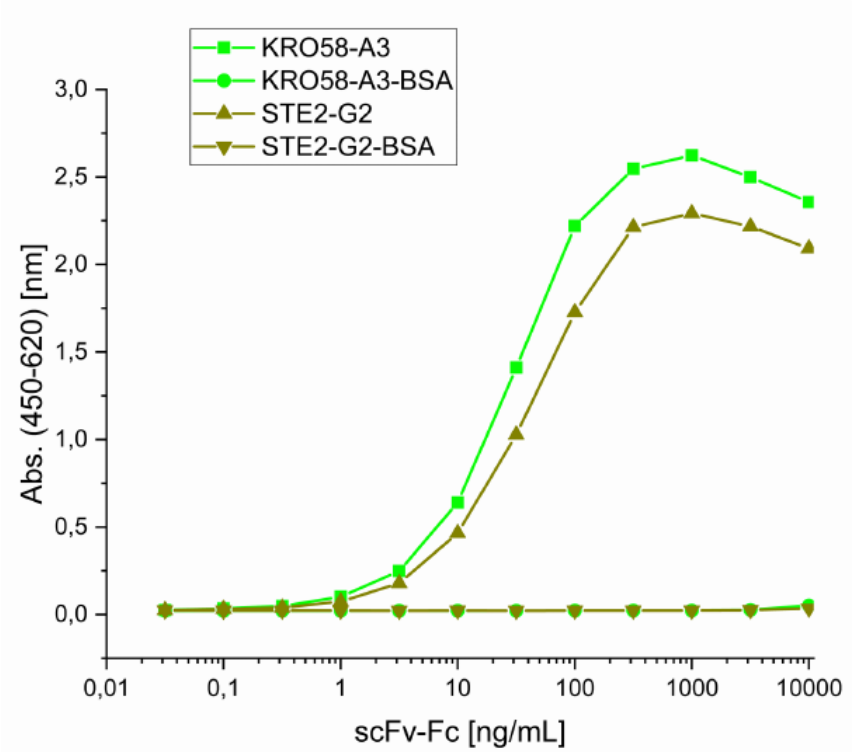


Supplemental Figure S4. Analysis of deletions in *Bra j IB* in mutated T_0 lines. (a) Lines carrying large deletions. PCR fragments from T_0 lines (T0-1, T0-21, T0-47, T0-55, see Figure S3) that were shorter than the expected size were subcloned and analyzed by Sanger sequencing and aligned to wild type sequences. x2: two clones were sequenced, x4: four clones were sequenced. sgRNA targeting sites are indicated by scissors, PAMs are underlined. WT: wild type. **(b)** Mutation analysis in two selected T_0 lines with *Bra j IA* and *Bra j IB* both mutated. PCR products from T_0 lines (T0-22, T0-32) were sequenced and chromatograms were shown below the wild type sequences. ICE decoded results were shown under the chromatograms.



Supplemental Figure S5. Analysis of the *Bra j I* homoeologs of T_1 offsprings to screen for inheritance of the deletion from the T0-1 line. Genomic DNA of 35 T_1 offsprings from the T0-1 mustard line which carried a heterozygous deletion of 695 bp at the *Bra j IB* locus was used for PCR amplification with homoeolog-specific primers to amplify *Bra j IA* and *Bra j IB* independently. No small fragment indicating a deletion was found. WT: wild type CR2664.

Supplemental Figure S6. Amino acid sequences alignment of Bra j I homoeologs in B. juncea varieties Terratop and CR2664. Bra j I specific antibodies (KRO58-A3 and STE2-G2) binding peptides *are* indicated by red lines.

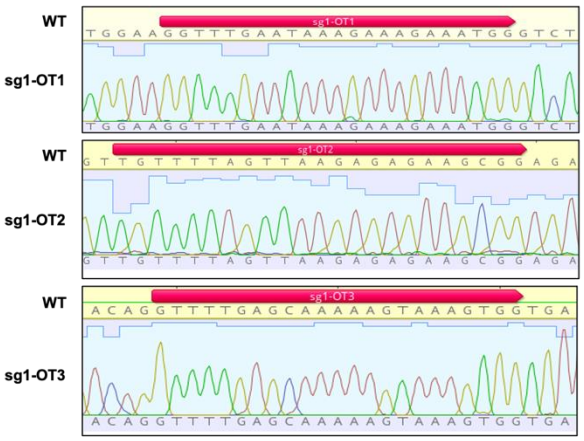


Supplemental Figure S7. Titration-ELISA for EC50 determination on Bra j I-peptide.

Supplemental Figure S8. Off-target detection. Two T_1 lines were selected for off-target mutation assay. The potential off-target sites (OT) were listed in the tables, mismatched sequences were highlighted in red, PAMs were highlighted in green. Sanger sequencing chromatograms from off-target sites (OT) were aligned to the wild type (WT) sequences. Off-score: predicted off-target efficiency score by CRISPR-P. sgRNA binding sites were indicated by red arrows upon the wild type sequences.

T1 line	Site	Sequence	off-score	Locus	Gene	Off-target mutation
31-5	sg1	GGTTTGAGTAAAGAGTGAAGCGG	-	A01:+12167892 B05:+14300539	-	-
	sg1-OT1	GGTTTGAATAAAGAAAGAAATGG	0.802	A08:-19115387	Intergenic	not detected
	sg1-OT2	TGTTTTAGTTAAGAGAGAAGCGG	0.535	A10:-12096893	BjuA038760	not detected
	sg1-OT3	GTTTTGAGCAAAAAGTAAAGTGG	0.506	B05:+31067798	Intergenic	not detected
	sg2	TTGTTTGTATTGATGAGTTTTGG	-	A01:+12167871 B05:+14300518	-	-
	sg2-OT1	TTGTTTGTGATGATGAGTTTTGG	0.571	A02:+1603829	Intergenic	not detected
	sg2-OT2	TTGTTTGTTATGATGAGTTTTGG	0.514	A01:-10693522	Intergenic	not detected
	sg2-OT3	TAGCTTATATTAATGAGTTTAGG	0.511	B06:+25658984	Intergenic	not detected
	sg5	GGTTTTATCTAGGATGTTTGAGG	-	A01:-12167205 B05:-14299843	-	-
	sg5-OT1	GGTCTTATCTAGGATGTTTGAGG	0.647	A01:+10676374	Intergenic	not detected
	sg5-OT2	TGTTTTAAATAAGATGTTTGTGG	0.640	B07:+20555103	Intergenic	not detected
	sg5-OT3	GGTTTTAGTTAAGATGTTTGAGG	0.599	A05:-14857022	Intergenic	not detected
	sg6	TAATATGTAAGGTTTTATCTAGG	-	A01:-12167215 B05:-14299853	-	-
	sg6-OT1	TAACATGGATGGTTTTATCTTGG	0.419	B04:-19577970	BjuB029221	not detected
	sg6-OT2	TCTTATGGAAAGTTTTATCTGGG	0.407	B08:-37894253	Intergenic	not detected
	sg6-OT3	TAATCTATAATATTTAATCTAGG	0.368	A05:+295433	Intergenic	not detected

T1 line	Site	Sequence	off-score	Locus	Gene	Off-target mutation
22-1	sg3	CACAAGCAAGCAATGCAGTCTGG	-	A01:-12167638 B05:-14300274	-	-
	sg3-OT1	CCAAAACAAGCAGTGAAGTCAGG	0.681	A06:+24547169	Intergenic	not detected
	sg3-OT2	CACAAGCAGGCAATGCAGTCCGG	0.667	A03:-4688191	BjuA041894	not detected
	sg3-OT3	CACAAGCAGGCAATGCAGTCCGG	0.533	A01:+10693765	BjuA004642	not detected
	sg4	CTTGCTTGTGGAGCCATTGCTGG	-	A01:+12167651 B05:+14300298	-	-
	sg4-OT1	CCTGCTTGTGGAGCCATTGCTGG	0.857	B05:+6926000	BjuB040397	not detected
	sg4-OT2	CCTGCCTGTGGAGCCATTGCTGG	0.779	B05:+6932255	BjuB040396	not detected
	sg4-OT3	CTTGCATGTGGAGCCATCGCTGG	0.371	B08:+69820088	BjuB005040	not detected
	sg7A	TGCCTCCATCTATCGGACGGTGG	-	A01:-12167771	-	-
	sg7A-OT1	CTCTTCCATCTATCAGACGGTGG	0.411	A08:-24369953	BjuA043849	not detected
	sg7A-OT2	GGCCGCCATTTATCTGACGGAGG	0.082	A09:+45546749	BjuA034058	not detected
	sg7A-OT3	TGCCTCACTCTAGCGGAAGGAGG	0.045	B08:-37511618	BjuB017257	not detected
	sg7B	TGCCTCCATCTACCGGACGGTGG	-	B05:-14300423	-	-
	sg7B-OT1	TGCCTCCGACTACTGGAAGGTGG	0.269	A03:-9847684	Intergenic	not detected
	sg7B-OT2	TGCCTCACTCTAGCGGAAGGAGG	0.024	B08:-37511618	BjuB017257	not detected
	sg7B-OT3	TGCCTCCATTTACCGGAGAGAAG	0.023	A06:-9768489	Intergenic	not detected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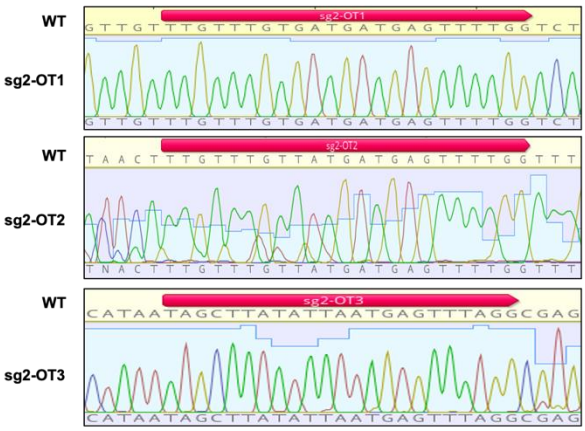
sg1-OT1
WT
sg1-OT2
WT
sg1-OT3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sg2-OT2
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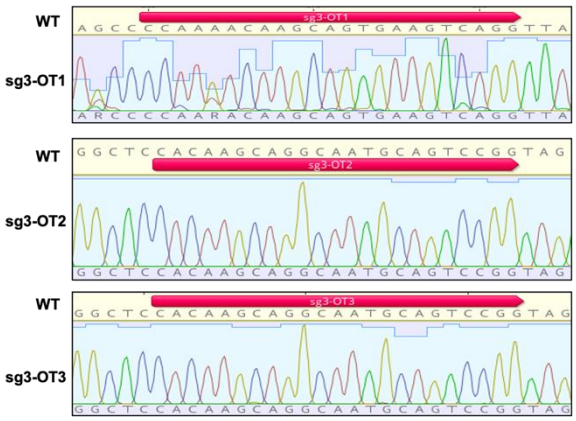
sg2-OT3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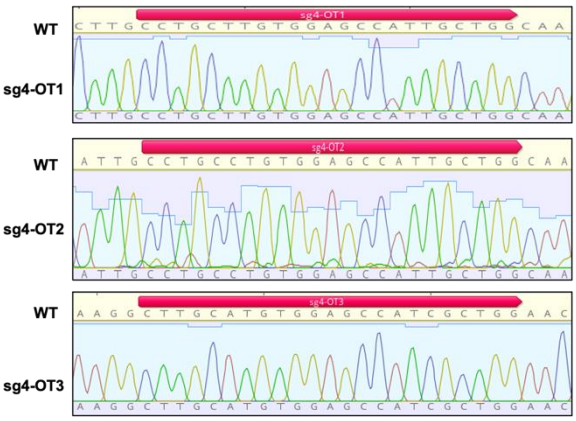
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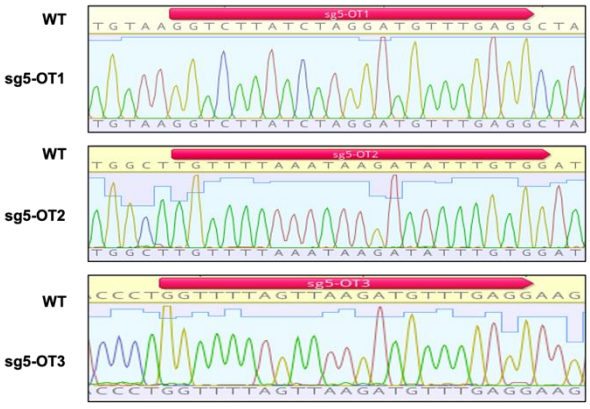
sg4-OT3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sg5-OT2
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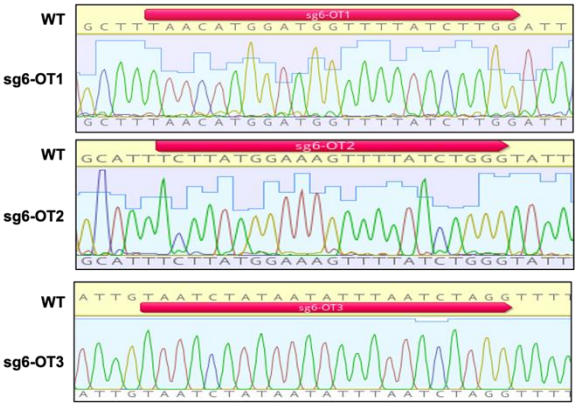
sg5-OT3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sg6-OT1
WT
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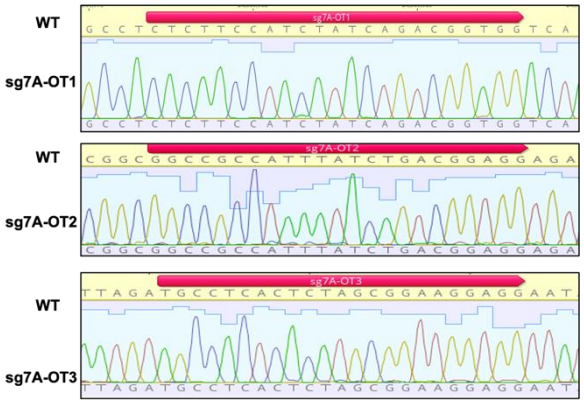
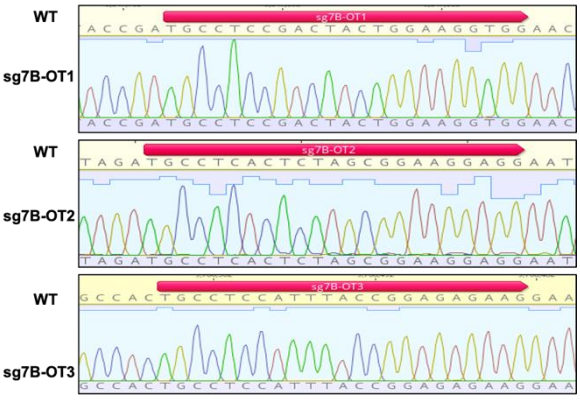
sg6-OT3


WT
sg7A-OT1
WT
sg7A-OT2
WT
sg7A-OT3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WT
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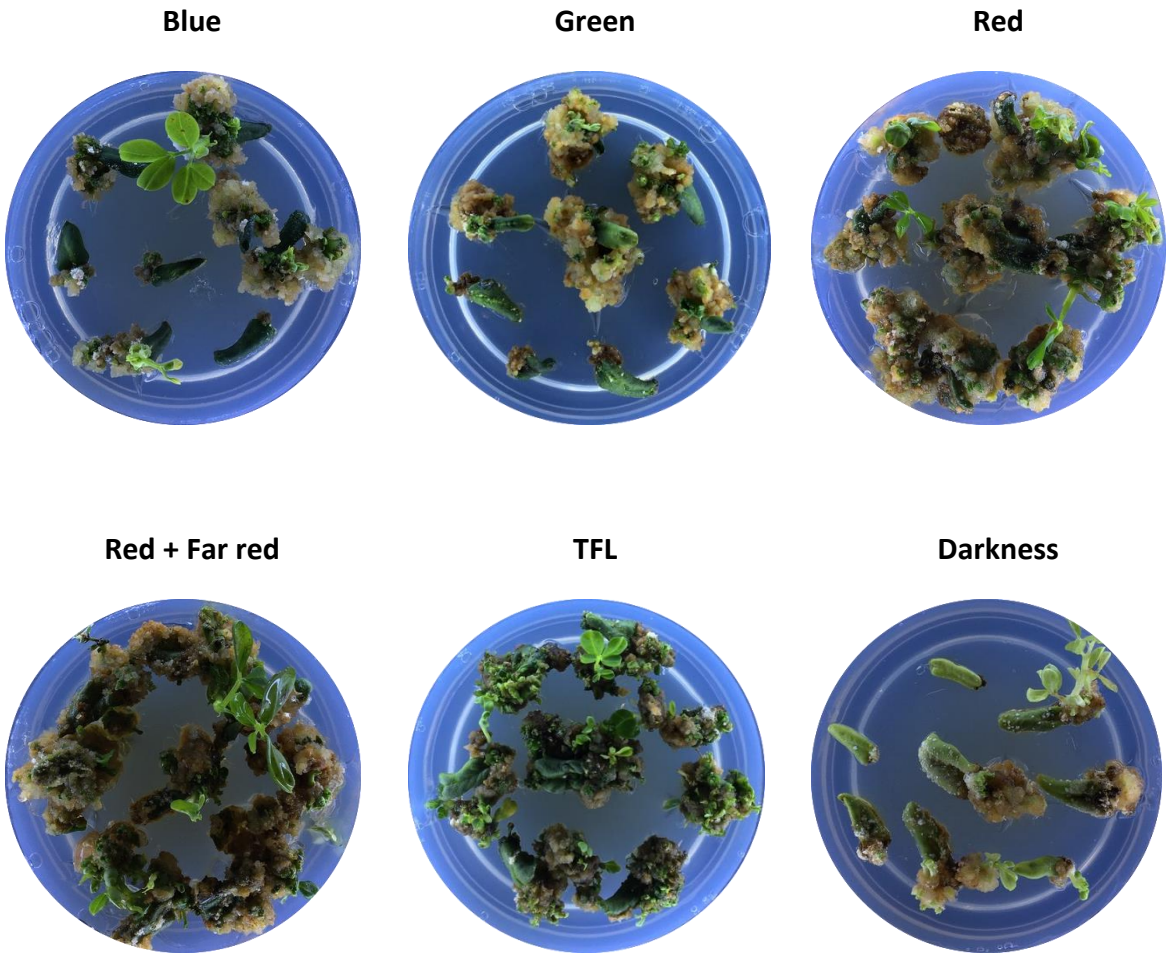
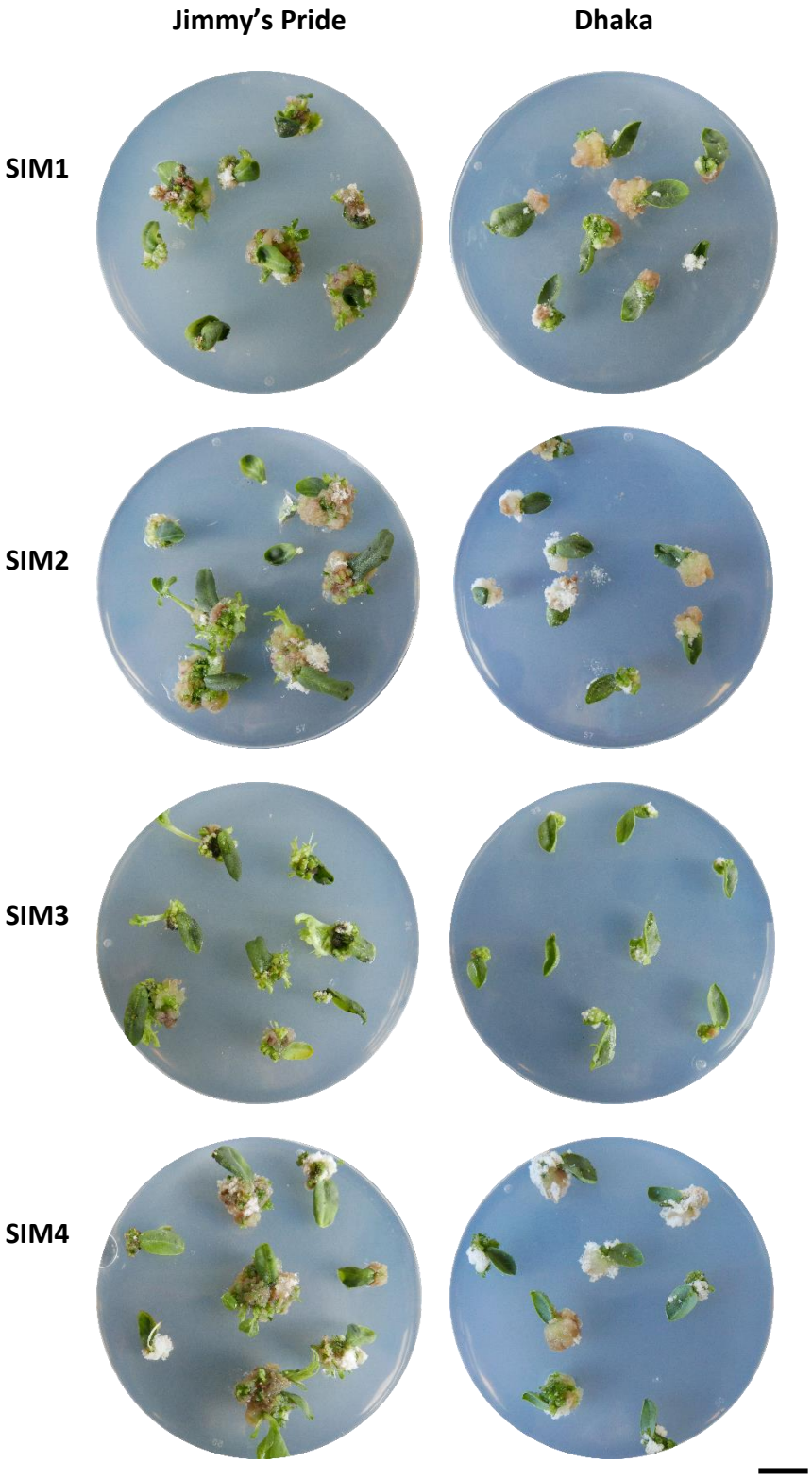
sg7B-OT3

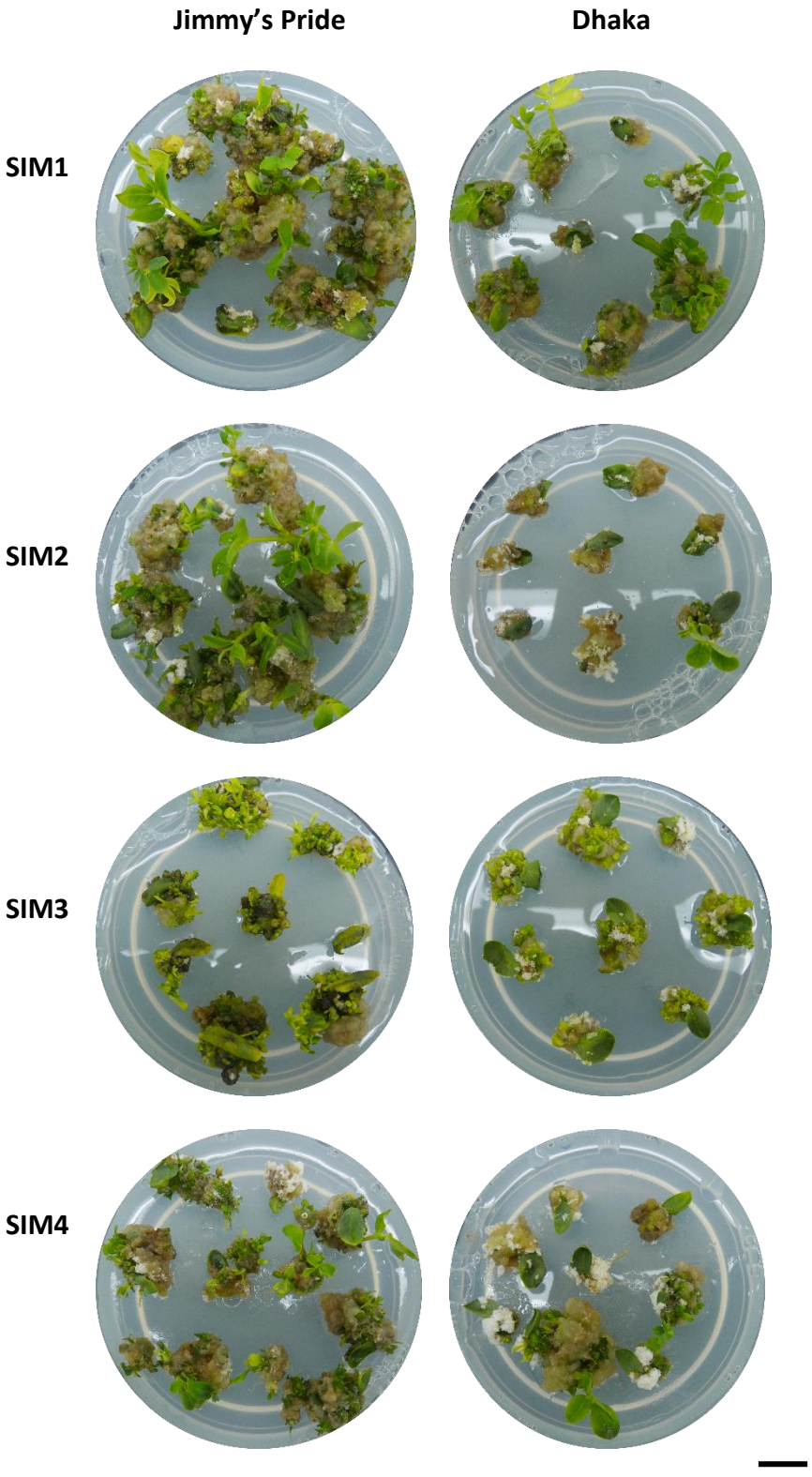
Supplemental materials for chapter 4



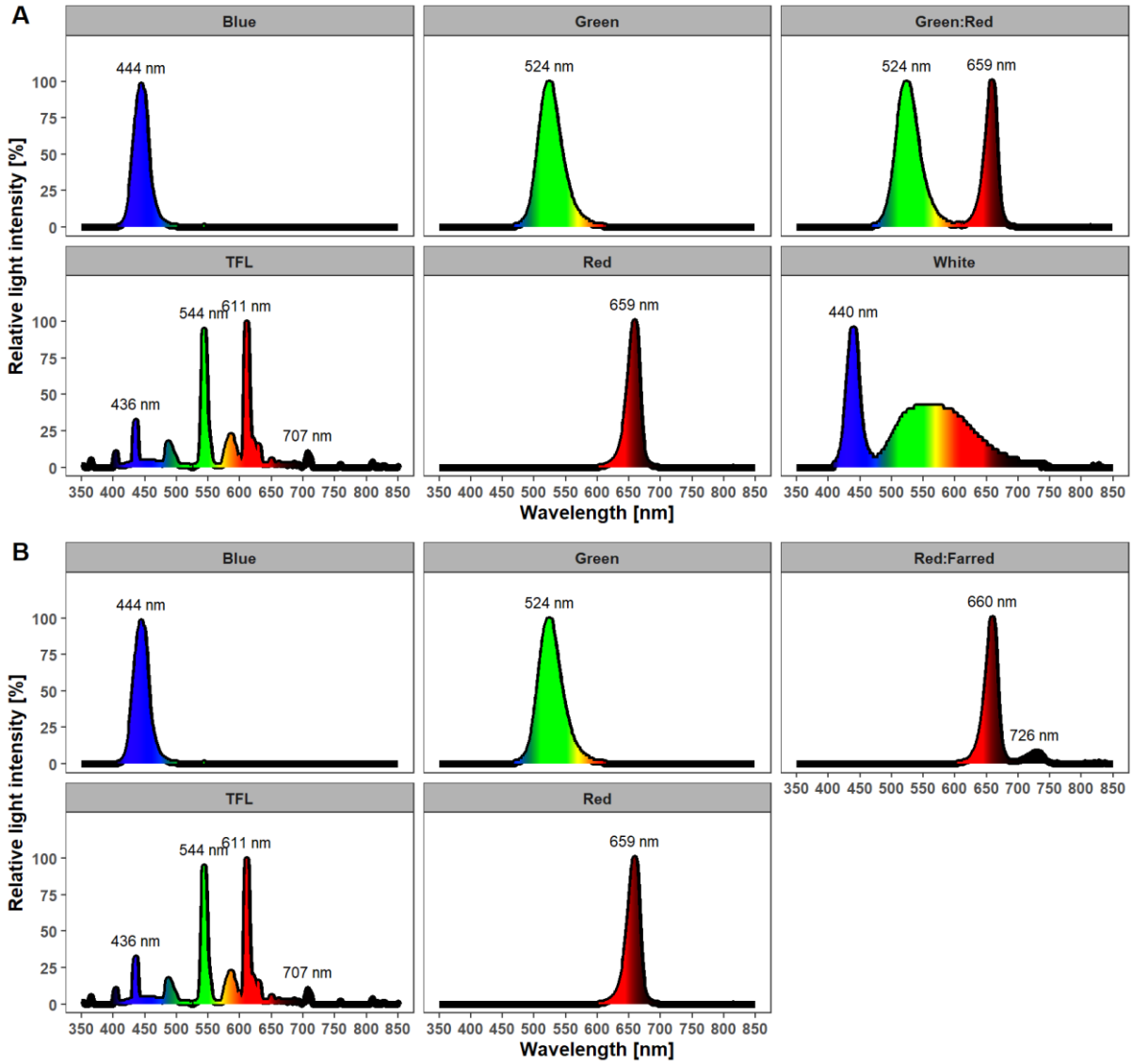
Supplemental Figure S1. Effect of different spectral qualities of light under controlled temperature on adventitious shoot regeneration from primary leaf explants of the line of the *Arachis hypogaea* line Jimmy's pride (Experiment 3). MS salts and vitamins were used in the media and explants were cultivated for 16 weeks on SIM1: 22.19 µM BAP + 2.3 µM Kin, TFL: tubular fluorescent lamp. Bar = 1 cm



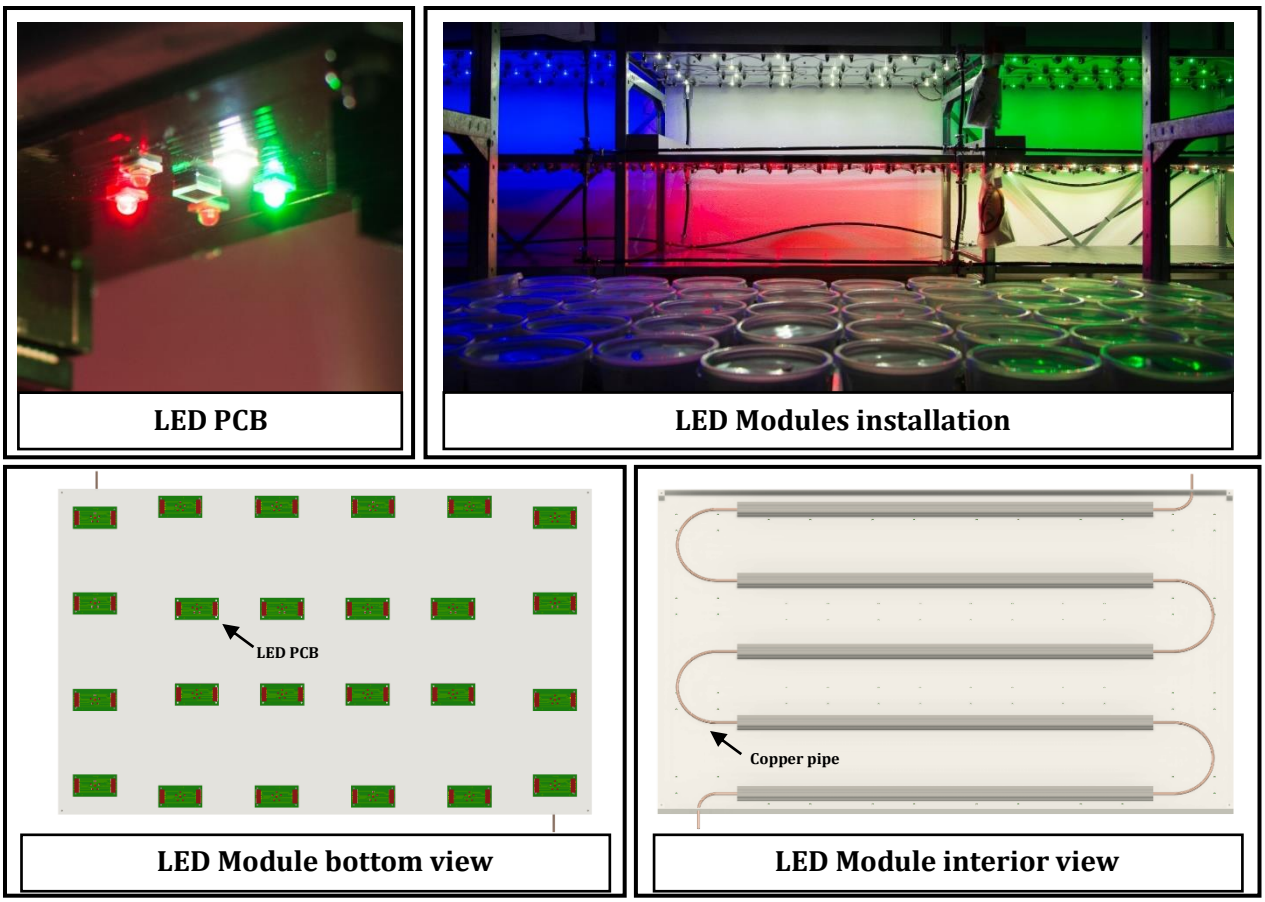
Supplemental Figure S2. Effect of cytokinins on adventitious shoot regeneration from primary leaf explants of two lines of *Arachis hypogaea* (Jimmy's pride and Dhaka). MS salts and vitamins were used in all media and explants were cultivated for 8 weeks on the different media SIM1: 22.19 µM BAP + 2.3 µM Kin, SIM2: 22.19 µM MT + 2.3 µM Kin, SIM3: 22.19 µM TDZ + 2.3 µM Kin and SIM4: 22.19 µM Zea + 2.3 µM Kin). Bar = 1 cm



Supplemental Figure S3. Effect of cytokinins on adventitious shoot regeneration from primary leaf explants of two lines of *Arachis hypogaea* (Jimmy's pride and Dhaka). MS salts and vitamins were used in all media and explants were cultivated for 12 weeks on the different media SIM1: 22.19 µM BAP + 2.3 µM Kin, SIM2: 22.19 µM MT + 2.3 µM Kin, SIM3: 22.19 µM TDZ + 2.3 µM Kin and SIM4: 22.19 µM Zea + 2.3 µM Kin). Bar = 1 cm



Supplemental Figure S4. Overview of the relative light intensity distribution and the wavelengths of the different light variants used in the experiment 2 (A) and experiment 3 (B).



Supplemental Figure S5. Overview of the growth chamber with the specific equipment.

Curriculum Vitae

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Academic Career

01/2018 – 02.2023	Ph. D. student at the Gottfried Wilhelm Leibniz-Universität Hannover, Hannover, Germany Institute of Horticultural Production Systems, Section of Woody Plant and Propagation Physiology Ph. D. thesis in the group of Prof. Dr. Traud Winkelmann
10/2015 - 09/2017	M. Sc. student at the Gottfried Wilhelm Leibniz-Universität Hannover, Hannover, Germany Institute of Horticultural Production Systems, Section of Woody Plant and Propagation Physiology Master thesis: Adventitious shoot formation and genetic transformation in *Dionaea muscipula* (Prof. Dr. Traud Winkelmann)
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10/1998 - 07/2006	Secondary school education, College Martin Luther King, Benin

List of publications

Peer review:

Assou, J., Zhang, D., Roth, K.D., Steinke, S., Hust, M., Reinard, T., Winkelmann, T. and Boch, J., (2021). Removing the major allergen Bra J I from brown mustard (*Brassica juncea*) by CRISPR/Cas9. *The Plant Journal*, *109*(3), pp.649-663, doi:10.1111/tpj.15584.

Assou, J., Bethge, H., Wamhoff, D., & Winkelmann, T. (2022). Effect of cytokinins and light quality on adventitious shoot regeneration from leaflet explants of peanut (*Arachis hypogaea*). *The Journal of Horticultural Science and Biotechnology*, 1-18.

Assou, J., Wamhoff, D., Rutzen, L. and Winkelmann, T., (2023). Optimization of in vitro adventitious shoot regeneration in *Brassica juncea* L. of different origins for application in genetic transformation and genome editing. *European Journal of Horticultural Science* (Accepted for publication).

Poster presentation:

Assou, J. Patzer. L., Rutzen, L., Schneider, E. and Winkelmann, Traud., (2019). Optimierung der Adventivsprossregeneration in vitro als Basis für die Etablierung von Transformationsprotokollen bei Erdnuss und Braunen Senf. DGG 53. Gaterbauwissenschaftliche Taggung, ‘’Future food production’’ 06-09. 03.2019, Berlin.

Assou, J., Zhang, D., Boch, J., and Winkelmann, T., (2020). Establishment of regeneration and transformation protocol to create hypoallergenic peanut and mustard through genome editing. International Symposium on Horticulture in Europe, Virtual SHE symposium, 09-11. 03. 2021.

Oral presentations:

Assou, J., Winkelmann, T., (2020). Utilization of optimized regeneration and transformation system to edit genes coding for allergenic seed storage proteins of *Brassica juncea* and *Arachis hypogaea.* 10. WeGa Doktorandentag, 01-02. 10. 2020 online.

Assou, J., Winkelmann T., (2021). Establishment of genetic transformation system of *Brassica juncea* to edit genes coding for allergenic seed storage proteins Bra J I. 11. WeGa Doktorandentag, 04-05. 02. 2021, Hannover.

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