Genetic transformation and regeneration in the Galena hop variety, towards CRISPR/Cas9 modification of shoot elongation

by

Katherine Sims

B.Sc., Simon Fraser University, 2018

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Summer 2022

Declaration of Committee

Name:	**Katherine Sims**
Degree:	**Master of Science**
Title:	**Genetic transformation and regeneration in the Galena hop variety, towards CRISPR/Cas9 modification of shoot elongation**
Committee:	**Chair:** **Leah Bendell** Professor, Biological Sciences
	Jim Mattsson Supervisor Associate Professor, Biological Sciences
	Sherryl Bisgrove Committee Member Associate Professor, Biological Sciences
	Charitha A. Jayasinghege Examiner Research Scientist, Crop and Plant Physiology Agriculture and Agri-Food Canada Agassiz Research and Development Centre

Abstract

Hop has separate female and male individuals, and self-fertilization cannot be used to generate plants homozygous for recessive alleles and traits, inhibiting breeding efforts. A long-sought trait is semi-dwarfism, as hop bines require tall trellises and specialized harvesting equipment. Recently developed CRISPR/Cas9 genome editing technology can be used to generate homozygous recessive mutant alleles, potentially overcoming this issue, but typically requires initial plant transformation and regeneration of transgenic plants – technologies that are inefficient and variety-specific in hop. Here we demonstrate the first example of (1) genetic transformation and shoot regeneration in the popular hop cultivar Galena and (2) improved transformation in hop through the ectopic expression of Arabidopsis BABYBOOM and WUSCHEL transcription factors. In addition, targets for gene editing of semi-dwarfism were identified and CRISPR cassettes for loss of function genome editing were generated and introduced into the Galena variety. Transformants are currently being evaluated based on genotype and stature.

Keywords: Hops; semi-dwarfism; shoot regeneration; plant transformation; gibberellin; CRISPR

Dedication

This thesis is dedicated to my husband Andrew and my two children, Taylor and Madison. Thank you for all the love and support you have given me through this incredible journey. You have shown me that it is never too late to pursue your passion.

Acknowledgements

I would like to express my sincere thanks and appreciation to my Professor, Jim Mattsson, for his constant encouragement and support with this research. Thank you to Sherryl Bisgrove, who was willing to contribute her time and expertise as a member of my Supervisory Committee. A debt of gratitude is owed to Mathias Schuetz for his guidance, generosity, and support with this project. I would also like to acknowledge Gisele Passaia from Willow Biosciences for her mentorship and invaluable advice. Special thank you to Michelle Messmer, Brendan Morris-Reade, Natalie Krumpa and Keiran Maskell for their assistance with this research. Lastly, I would like to acknowledge all the students, present and past, that I have had the pleasure of sharing a lab with. Your encouragement and friendship will always be cherished.

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

1.1. Humulus lupulus

Humulus lupulus (the common hop) is an herbaceous liana, indigenous to the temperate climates of the Northern hemisphere. This enthusiastic climber develops an extensive perennial root system that gives rise to annual shoots. Emerging shoots climb in a clockwise direction and can reach over six meters in length in a single growing season (Neve, 2012). Leaves are typically arranged in pairs, opposite one another, consisting of 3-5 lobes with serrate margins. The species is normally diploid (2n=20) and dioecious, meaning there are separate male and female plants. Monoecious individuals have been identified, but are predominantly triploid due to a failure of paternal gamete reduction (Skof et al., 2012). The economic importance of the hop plant lies with the female inflorescence specific to *H. lupulus var. lupulus*. Female flowers, often referred to as strobili or hops, have cone-like architecture consisting of overlapping bracts that connect to a central margin. As the flower ripens, the inner bracts develop an abundance of glandular trichomes called lupulin glands that sequester resins, oils and polyphenols commonly used in the pharmaceutical and brewing industries. Male flowers are small and occur in multibranched loose panicles. They develop few resin glands in contrast to large quantities of wind-borne pollen. As such, hop farms are typically devoid of male plants and only females are propagated and cultivated for commercial use.

1.1.1. Commercial use

For centuries, hop bines of *H. lupulus L.* have been collected for their antimicrobial, anti-inflammatory, anti-depressant, and sedative properties. These traditional claims have been thoroughly supported by several pharmacological studies (Abram et al., 2015; Benkherouf et al., 2020; Hall et al., 2008; Mizobuchi & Sato, 1985; Zanoli et al., 2005) and new therapeutic uses such as chemoprevention and oestrogenicity have been identified (Milligan et al., 2002; Miranda et al., 1999; Tyrrell et al., 2010; Zierau et al., 2002). Despite increasing reports of medicinal potential, hop is more well known as an essential ingredient in beer, along with wheat, barley, and yeast. Functioning originally as a natural preservative, use of the female hop cone as a flavoring agent grew in popularity throughout the Middle Ages to the present day. Currently, 98% of hop is grown exclusively for the brewing industry (Korpelainen &

Pietiläinen, 2021) with production dominated by Germany and the North-West United States: Washington, Oregon, and Idaho (Hop Growers, 2018). There are now close to 300 industrial cultivars established, classified based on chemical composition and flavoring profiles (*Hopslist | The Home of Beer Hops*, *2017*). Important varieties traded in the world market include the bittering favorites; Magnum, Taurus, Herkules, Galena, Nugget, Millennium and CTZ, while aromatic representatives include Perle, Spalter Select, Mittelfrüh, Hersbrucker, Tettnanger, Saaz and Cascade (Almaguer et al., 2014).

1.1.2. Novel cultivar establishment

There is a continued desire to advance hop cultivation by improving agronomic traits such as disease resistance, secondary metabolite content, vegetative behavior, and storage stability. The development of a novel hop cultivar involves repeated genetic crosses between selected female and male plants, and selection of offspring, typically based on incrementally improved phenotypes of interest. Finally, identified preferred individuals are clonally propagated by vegetative cuttings to maintain their genotypes (Turner et al., 2011). In most cases, offspring from a genetic cross contain unwanted traits from both parental lines, requiring repeated backcrossing to restore the preferred genomic background (Beatson et al., 2003). This process is both expensive and time consuming (Morris & Bellon, 2004). As many commercial hops have been bred from similar parental lines or selected through clonal variation, there is an evident narrow range of genetic diversity in economically important varieties (Jakse et al., 2004; Murakami et al., 2006; Patzak et al., 2010). Moreover, traditional genotype selection has been based on plant morphology and chemical composition rather than molecular markers; traits that are easily influenced by environmental factors (Korpelainen & Pietiläinen, 2021).

Cross pollination of industrial cultivars with wild hop populations has become an attractive approach to overcome genetic limitations (Neve, 2012). Molecular analyses, based on microsatellites (Jakse et al., 2004; Murakami et al., 2006; Patzak et al., 2010), gene-specific markers (Bassil et al., 2008) and amplified fragment length polymorphisms (Townsend & Henning, 2009) demonstrate how wild hop has maintained a high degree of allelic variability in contrast to modern landraces, offering an unexploited genetic resource for hybridization. As hop is highly heterozygous, meaning the plant often contains two different alleles at a given locus, resulting offspring have a mix of alleles

capable of producing phenotypes distinguishable from both parents. This can be seen as advantageous if the goal of the breeder is to establish a cultivar with a combination of unique traits; however, if there is a want to introduce specific adaptations into a given individual, a more targeted approach is required.

Genetic transformation is often an attractive alternative to conventional breeding, as it allows for the introduction of desirable characteristics without altering the background genotype. In plants, transformation is generally mediated by the bacteria *Agrobacterium tumefaciens*, a.k.a. *Agrobacterium radiobacter*, which can transfer a segment of DNA, transfer (T)-DNA, from a plasmid into the nucleus of infected cells, where it subsequently gets integrated into the host genome. Although successful in many species, numerous obstacles have discouraged the use of genetic transformation of hop, mainly a recalcitrance to *in vitro* shoot regeneration and toxicity of agents used to select for transformation events. Consequently, few examples of published procedure are available, each of which include a limited number of genotypes that differ in requirements for obtaining transgenic individuals. Most reports demonstrate proof-of-concept through the introduction of the *gusA* reporter gene (Batista et al., 2008; Gatica-Arias & Weber, 2013; Horlemann et al., 2003; Okada et al., 2003; Škof & Luthar, 2005), while others involve the integration of expression cassettes that enhance secondary metabolite production (Mishra et al., 2018; Schwekendiek et al., 2005). In the former, transient expression and chimerism of transgenes were identified (Batista et al., 2008; Škof & Luthar, 2005). Other examples demonstrate stable integration, specifically in genotypes Tettnanger (Horlemann et al., 2003) and Osvald-72 (Mishra et al., 2018; Okada et al., 2003), showing promise for future endeavours. As stable transformation success has been met with low transformation efficiency, original protocols require optimization. Furthermore, additional procedures need to be identified for alternative genotypes.

1.2. Plant Regeneration

Throughout plant development a variety of somatic cells maintain a degree of totipotency, allowing for phenotypic plasticity and regenerative responses. Organ regeneration can be observed in several forms, which varies according to species and genotype. Humans have exploited this totipotency for a long time, primarily by regeneration of whole plants from shoot, leaf, root and cane cuttings. Regeneration has

also been observed on a tissue level. Within days of wounding of a stem, healing of a damaged epidermis begins, and the development of new vessel elements and sieve tubes re-establish disrupted vascular connections around the site of injury (Benayoun et al., 1975). This process involves the deposition of suberin, lignin and soluble waxes in the epidermis (Savatin et al., 2014) as well as the coordinated rearrangement of PIN1 proteins and polar auxin flow allowing for directional vascular differentiation in the stem (Aloni, 2010; Mazur et al., 2016). Under strict tissue culture conditions involving hormone treatments, many otherwise recalcitrant plant species are capable of full body and organ regeneration (Ikeuchi et al., 2016, 2019), procedures that can be used for mass propagation and also in conjunction with genetic transformation to regenerate transgenic plants.

Regeneration can proceed indirectly via an intermediate callus phase or directly from somatic cells. Of the two developmental routes, indirect regeneration occurs more frequently, but differentiating between the two mechanisms can be difficult, as they often occur simultaneously on the same explant (Horstman, Bemer, et al., 2017). Somatic embryogenesis, an example of complete totipotency, is used to describe the formation of an embryo that develops from a somatic cell lineage as opposed to gamete fusion. Somatic embryos can form on vegetative explants following exposure to extreme environmental stressors or high levels of auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D) (Merkle et al., 1995). More recently, manipulation of embryo-specific gene expression has served as a means for somatic embryo induction and has shed light on molecular aspects surrounding plant regeneration (Gordon-Kamm et al., 2019). Organogenesis is used to describe pluripotent plant responses. When initiated, ectopic meristems grow to give rise to either new shoots or roots. Somatic cells capable of forming whole organs do not appear to have the same competence of cells that pursue embryo development (Verdeil et al., 2007). Embryos have characteristic bipolarity, with apical and basal poles distinguishing the future shoot and root, respectively, while organogenesis results in the expression of unipolar structures that maintain a lignified vascular connection to the underlying explant (Horstman et al., 2017).

1.2.1. De novo shoot organogenesis

The underlying basis for organ regeneration is highly dependent on the antagonistic actions of two key plant hormones, auxin and cytokinin. Generally, high cytokinin to auxin ratios specify shoots, low cytokinin to auxin ratios give rise to roots and an even ratio results in a mass of proliferating cells called callus (Skoog & Miller, 1957). A common method for inducing shoot organogenesis consists of exposing cultured explants to 2,4-D to stimulate callus formation, followed by a transfer to cytokinin-rich media to promote the development of shoots (Christianson & Warnick, 1983). Callus derived from both above and below ground organ explants forms through a series of xylem-pole pericycle asymmetric divisions via a pathway that resembles lateral root meristem (LRM) initiation (Atta et al., 2009; Sugimoto et al., 2010). Several molecular markers specific to the LRM are expressed during callus development (Table 1) (Atta et al., 2009; Che et al., 2007; Laplaze et al., 2005), rejecting original hypotheses that callus is unspecialized and derived from de-differentiated cells. When callus is selected and sub-cultured on to cytokinin-rich media, founder cells of LRM-like tissue experience a mitotic pause, followed by a change in differential gene expression, favoring a LRM to shoot apical meristem (SAM) transition (Rosspopoff et al., 2017). Organ commitment is engaged once cell divisions resume. The establishment of SAM, followed by the appearance of leaf primordia occurs from a population of adjacent cells indicating regeneration requires positional information, opposed to a single cell event (Subban et al., 2021). Early inductive cues involved in shoot regeneration occur within the first four to five days following incubation on cytokinin-rich media (Subban et al., 2021). Premature removal of calli off hormone supplementation, results in a failure to produce shoots, indicating a crucial window for initiation.

1.2.2. Cytokinins

Cytokinins are N^6-substituted purine derivatives that play a central role in a variety of physiological events, including shoot formation, floral organ development, root inhibition, cell division (cytokinesis), apical dominance and leaf senescence (reviewed in Kieber & Schaller, 2014; Wybouw & De Rybel, 2019). Naturally occurring cytokinin is synthesized in both root and shoot cells. Root cytokinin, predominantly zeatin-type, are actively pumped into the xylem and translocated to the upper extremities, where it is hypothesized to function as a long-distance signal coordinating root and shoot

development (Kudo et al., 2010; K. Zhang et al., 2014). Cytokinin synthesized in the shoot, primarily isopentenyladenine (iP), are transported through symplastic connections in the phloem, where they regulate auxin transport and vascular patterning in the root meristem (Bishopp et al., 2011; Hirose et al., 2008; Kudo et al., 2010). Plants respond to the presence of cytokinin upon binding to histidine-kinase receptors, which ultimately activates two types of response regulators (RR) that target downstream effectors in the nucleus (reviewed in Kieber & Schaller, 2018). The plant can regulate cytokinin activity either through sugar conjugation or degradation via cytokinin oxidase (CKX). The importance of cytokinin input during *in vitro* shoot regeneration has been highlighted in several studies, with outcomes dependent on species, genotype and cytokinin content (Coleman & Ernst, 1989; S. C. Debnath, 2005; Hunkova et al., 2016; Magyar-Tábori et al., 2010; Mok et al., 1980). Functional characterization has predominantly come from studying the effects of exogenous cytokinin applications or manipulation of endogenous cytokinin levels.

Zeatin, which was isolated from the endosperm of immature corn, was the first naturally derived cytokinin identified (Letham, 1973). Like all endogenous forms, it contains an isopentenyl side chain attached to the N^6 position of its adenine ring. It occurs in both *cis* and *trans* configurations in higher plants; *trans* being the active form (Gajdošová et al., 2011). The side chains of synthetic cytokinins, such as kinetin and 6-benzylaminopurine (BAP), contain aromatic ring substitutions making them resistant to enzymatic cleavage via CKX, increasing their stability in culture (Kieber & Schaller, 2014). Both Zeatin and BAP have proven to be effective at promoting shoot regeneration, shoot proliferation and rooting in *in vitro*, with reports including various species and explant choice (C. S. Debnath, 2006; García-Fortea et al., 2020; Moura et al., 2009; Passey & Jones, 1983). Depending on the genotype, either cytokinin can be more advantageous over the other. For example, zeatin was shown to stimulate shoot initiation in *Populus deltoides* (poplar), while BAP was deemed inhibitory and phytotoxic (Coleman & Ernst, 1989). In contrast, BAP is considered the cytokinin of choice for shoot organogenesis in apple (Magyar-Tábori et al., 2010) and successfully replaced zeatin use in micropropagation systems in *Olea europaea* (olive) (Peixe et al., 2007). Differential responses are likely an outcome of species-specific uptake, transfer, metabolism, and cellular interactions with endogenous cytokinin.

The phenylurea derivative thidiazuron (TDZ), originally registered as a cotton defoliant, shares no structural similarity with cytokinin, but exhibits relatively high cytokinin-like activity (Ahmad & Faisal, 2018). TDZ has shown to be more effective than zeatin, kinetin and BAP when inducing callus growth, auxiliary shoot proliferation, organ regeneration and somatic embryogenesis (Genkov et al., 1995; Lu, 1993; Singh et al., 2016; J. C. Thomas & Katterman, 1986). Hence, TDZ has often become the PGR of choice for species that display recalcitrance in tissue culture. The underlying mechanism of how TDZ exerts a response is unclear, but evidence implies that it promotes purine cytokinin synthesis and suppresses CKX activity, allowing for endogenous cytokinin accumulation *in vivo* (Bilyeu et al., 2001; Pai & Desai, 2018). Several independent studies have also reported an association between TDZ presence and the metabolism of auxin, ethylene and abscisic acid (Ji & Wang, 1988; Lu, 1993; Murthy et al., 1996; Suttle, 1986). Although, TDZ has shown to be a potent regulator of plant growth and development, several drawbacks have been associated with its use in culture. Morphological defects such as root inhibition, fasciated shoots, abnormal leaf formation, unorganized vascular bundles and inhibition of shoot elongation have been reported following exposure (Dewir et al., 2018). Furthermore, high-dose applications or prolonged dosing have been shown to inhibit shoot formation in several species (Dewir et al., 2018; Huetteman & Preece, 1993). Therefore, following shoot organogenesis, subculture to a secondary media containing either a lower dose of TDZ or an alternative cytokinin source is often used.

1.2.3 Growth and regeneration enhancers

The use of growth and regeneration enhancers in plant culture has been well documented. Common enhancers include silver thiosulfate (Steinitz et al., 2010), silicon (Sivanesan & Park, 2014), coconut water (Parismoreno Rivas et al., 2019; Peixe et al., 2007), and gibberellic acid (Isogai et al., 2008). The non-ionic surfactant, Pluronic F-68 (PF-68), has been linked to cell growth and proliferation, nutrient uptake, and cellular repair in both plant and animal culture. Early studies using animal cell lines have shown that PF-68 offers protection against membrane shearing during aeration and agitation of liquid culture (Meier et al., 1999). Moreover, it has been demonstrated that supplementation stimulates 2-deoxyglucose uptake and amino acid incorporation into proteins (Cawrse et al., 1991). In yeast, it was revealed that PF-68 induces the formation

of transmembrane pores, increasing membrane permeability and improving nutrient uptake (King et al., 1991).

In plant tissue culture, PF-68 has shown to stimulate callus growth, promote protoplast proliferation and improve micropropagation in a variety of species. Treated callus in recalcitrant *indica* rice resulted in callus growth and increased protein biosynthesis, nitrogen metabolism and sugar and potassium uptake. (Kok et al., 2021). In similar studies, PF-68 promoted protoplast proliferation in red pepper (Kaparakis & Alderson, 2015) and cell division in transgenic tobacco (Lee & Kim, 2002). Key regenerative responses have been noted in both jute (Khatun et al., 1993) and citrus (Cancino et al., 2001). Regenerated plants exhibited no adverse side-effects and developed morphologically normal. Current studies are focusing on the mechanism behind the growth-stimulating quality of PF-68 as well as its influence on shoot regeneration in additional recalcitrant species.

1.2.4 Candidate genes for improving regeneration capacity

Mutant characterization in *Arabidopsis thaliana* has allowed for the identification of several gene candidates that participate in regenerative pathways. Candidates implicated in totipotent and pluripotent responses have been successfully introduced into a variety of model plants and important crops, showing promise for overcoming regeneration recalcitrance and improving transformation output (reviewed in Gordon-Kamm et al., 2019).

Expression of the homeodomain transcription factor *WUSCHEL* (*WUS*) is the earliest event that marks SAM stem cell initiation in *Arabidopsis* (Zhang et al., 2017). During *de novo* shoot regeneration *WUS* activation triggers the transition of shoot promeristem to shoot progenitors following Type-B ARABIDOPSIS RESPONSE REGULATOR (ARR)-mediated cytokinin signaling (Gordon et al., 2007; Negin et al., 2017; Sakai et al., 2001; Zhang et al., 2017). *WUS* expression defines the organizing center during SAM establishment and also participates in regulating the size of the stem cell niche through the activation of *CLAVATA3* (*CLV3*) in the central zone (Brand et al., 2000; Schoof et al., 2000; Yadav et al., 2011). *CLV3* encodes a secreted peptide that is perceived by the membrane localized receptor complex *CLV1/CLV2*, which upon binding, initiates a transduction cascade that downregulates *WUS* activity (Brand et al.,

2000; Schoof et al., 2000; Yadav et al., 2011). The *WUS-CLV3* negative feedback loop regulates the balance between stem cell replenishment and cell incorporation into shoot primordia. Ectopic expression of *WUS* has been reported to enhance shoot regeneration and improve transgenic plant recovery, even in recalcitrant species (Lowe et al., 2016). *WUS* is currently the only identified factor that regulates both pluripotent stem cells in the meristem and totipotent stem cells during embryogenesis (Elhiti et al., 2013). Interestingly, the loss-of-function mutant is still able to develop somatic embryos at low frequency, indicating the presence of alternative pathways that function during embryogenesis (Elhiti et al., 2013).

The *AINTEGUMENTA-LIKE* (*AIL*) family of *AP2/ERF* transcription factors play overlapping roles in embryogenesis, organ placement, stem cell specification and meristem development (reviewed in Horstman et al., 2014). The *Arabidopsis* AIL proteins include *AINTGUMENTA*, *BABYBOOM* and the *PLETHORA* genes. Because of genetic redundancy, *AIL* loss-of-function mutations fail to produce obvious phenotypes when single genes are knocked out; however, different *AIL* mutant combinations can result in meristem absence, loss of organ identity and embryo arrest (Aida et al., 2004; Galinha et al., 2007; Krizek, 2015; Mudunkothge & Krizek, 2012). When gene representatives are overexpressed, ectopic organ formation, callus growth and SE is induced (Horstman, et al., 2017). The AIL transcription factor BABYBOOM (BBM), originally identified in *B. napus*, is expressed in the embryo and root meristem where it regulates cell identity and pluripotent growth along with other AIL proteins (Boutilier et al., 2002; Horstman et al., 2014; Karim et al., 2018). The overexpression of endogenous and heterologous *BBM* genes has been linked to cell proliferation and differentiation, shoot organogenesis, somatic embryogenesis and the promotion of apogamy. The ability to induce somatic embryos when transiently expressed, has motivated the use of this gene as a genetic tool for improving plant transformation in a variety of important crops and recalcitrant species (reviewed in Jha & Kumar, 2018). Ectopic *BBM* expression has been reported to improve transformation output in *Arabidopsis* (Lutz et al., 2015), *Capsicum annuum* (Heidmann et al., 2011*)*, *Sorghum bicolor* (Lowe et al., 2016), *Saccharum officinarum* (Lowe et al., 2016*)* and *Oryza sativa* (Bui et al., 2017; Lowe et al., 2016).

The BBM protein transcriptionally activates members of the *Arabidopsis LAFL*/*AGL15* network: *LEAFY COTYLEDON1/2* (*LEC1/2*), *ABSCISIC ACID-*

INSENSITIVE3 (*ABI3*), *FUSCA3* (*FUS3*), *LEC1- LIKE* (*L1L*) and *AGAMOUS-LIKE15* (*AGL15*) (Horstman, et al., 2017). The *LAFL* genes redundantly function early during the acquisition of embryo identity and later during embryo maturation and dormancy (Jia et al., 2013). Ectopic expression of either *LEC1* and *LEC2* is sufficient for inducing the formation of somatic embryos on the cotyledons of *Arabidopsis* seedlings in the absence of exogenous growth regulators or stress treatments (Gaj et al., 2005; Lotan et al., 1998; Stone et al., 2001). A study conducted in *Arabidopsis* by Horstman *et al*. showed that both *LEC2* and *AGL15* positively regulates *BBM*-mediated embryogenesis and that *LEC1* and *FUS3* are essential for the process. Regenerative responses were shown to be dependent on both the dose of nuclear-localized BBM protein as well as the developmental stage of the tissue used in culture. Low to intermediate levels of BBM activity promoted the formation of ectopic leaves, while intermediate to high levels induced the formation of somatic embryos. BBM also appeared to initiate somatic embryogenesis in two ways: directly from cotyledons when BBM was activated prior to germination and indirectly following a callus phase when activated post-germination. Indirect somatic embryogenesis occurred slowly and independent of BBM-mediated *LEC1* input, suggesting LAFL gene targets are transcriptionally inaccessible until after callus formation. LAFL genes are epigenetically downregulated via chromatin remodeling proteins and their chromatin state may influence whether direct or indirect regeneration occurs. This also implies that BBM uses an alternative signalling pathway to induce indirect SE.

An additional number of proteins have been recognised to promote regenerative responses in culture. Genes involved in cytokinin signaling, epigenetic modifications, embryo development, and shoot and root meristem establishment are over-represented amongst identified genetic factors (Table A.1). The literature review by Ikeuchi et al., 2019 discusses recent advances in the molecular and cellular events that participate in shoot organogenesis and SE and serves as an excellent “search” resource for additional developmental genes that could function as tools for stimulating plant regeneration programs.

1.3. Gibberellin

Ubiquitous among higher plants, gibberellins (GAs) are comprised of a large group of diterpenoid carboxylic acids that participate in a diverse range of plant

developmental processes including; stem elongation, leaf expansion, SAM establishment, seed germination and flower development (reviewed in Thomas et al., 2005). Interestingly, GA was originally identified as secondary metabolites from the fungus *Gibberella fujikuroi*, after infections of rice seedlings caused severe stem overgrowth and sterility. Its profound effects on plant growth and development resulted in over a century of research (reviewed in Hedden & Sponsel, 2015). As of 2020, 136 GAs have been recognized across vascular plants, bacteria, and fungi, named $GA_{1\text{-}136}$ according to their discovery (Gao & Chu, 2020; MacMillan, 2001). The most famous GA-induced growth response is the induction of internode elongation in GA-deficient mutants (Hedden & Sponsel, 2015a; Sponsel & Hedden, 2010). However, it is evident from these assays, that only a small fraction of GA possesses intrinsic activity. These mainly include GA_1, GA_3, GA_4, and GA_7, each of which share the common molecular characteristics: a hydroxyl group on C-3β, a carboxyl group on C-6, and a lactone between C-4 and C-10 (Yamaguchi, 2008). Plants deficient in GA show phenotypes of dwarfism, prolonged germination, inhibition of root elongation and sterility.

1.3.1. Gibberellin-induced growth and elongation

GA promotion of organ growth is a result of enhanced cell elongation and division. GA induces transcript levels of genes involved in both processes. For example, the expression of genes encoding xyloglucan endotransglycosylases (XETs) were shown to increase in response to GA during leaf expansion in *Hordeum vulgare* (R. C. Smith et al., 1996) and internode elongation in *O. sativa (Uozu et al., 2000)*. XETs are enzymes that increase cell wall plasticity during cell elongation by reorganizing xyloglucan polymers in the cell wall (R. C. Smith & Fry, 1991). Furthermore, GA also upregulates the expression of expansins and pectin methyltransferases (PME) during *Arabidopsis* seed germination (Ogawa et al., 2003). Expansins cause loosening of the cell wall by disrupting hydrogen bonds between cellulose microfibrils and matrix polymers (Cho & Kende, 1997), while PME promotes cell wall loosening through pectin modifications (Ogawa et al., 2003). The loosening of cellulose microfibrils, running mostly perpendicular to the cell axis, allow for turgor-driven cell extension during shoot elongation. In deep water rice, GA was shown to stimulate rapid cell division in the shoot meristem at the G1/S check point (Lorbiecke & Sauter, 1998) and during G2/M phase progression. The later involving GA-induced accumulation of transcripts encoding the

regulatory and catalytic subunits of cyclin-dependent kinases, central to cell cycle regulation (Fabian et al., 2000). Microarray analyses in *Arabidopsis* demonstrated an upregulation of genes, *cyclinD* and *MCM*, also involved in G1/S transition, upon exposure to exogenous GA (Ogawa et al., 2003). The mechanisms behind GA-induction of these genes as well as crosstalk involving additional hormone signals still requires further understanding.

1.3.2. Gibberellin biosynthesis pathway

Plant GAs are synthesized from the common diterpenoid precursor, geranylgeranyl diphosphate (GGDP), which is predominantly derived from the methylerythritol phosphate pathway in the chloroplast (Kasahara et al., 2002). Biosynthesis can be divided into two parts, early and late, and involves three different classes of enzymes: terpene synthases, cytochrome P450 monooxygenases (CYP450s) and 2-oxoglutarate, iron-dependent dioxygenases (2ODDs) (Figure 1). In the chloroplast, cyclization of linear GGDP occurs in two steps by the terpene synthases *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). First, CPS converts GGDP to bicyclic *ent*-copalyl diphosphate, then KS catalyzes the formation of the tetracyclic compound *ent*-kaurene. Two CYP450s, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO), catalyze the conversion of *ent*-kaurene to *ent*-kaurenoic acid, which is then converted to GA_{12}, respectively (Helliwell et al., 1999). Protein-fusion experiments with green fluorescent protein suggests that KO is localized to the outer envelope of the chloroplast, while KAO is present in the endoplasmic reticulum (Helliwell et al., 2001).

GA_{12} lies at a branch-point between two parallel pathways and can either undergo C-20 oxidation, ultimately forming bioactive GA_4, or C-13 hydroxylation to produce the precursor GA_{53}, initiating the formation of GA_1. Genes encoding C13-hydroxylases have yet to be identified with certainty, but preliminary evidence suggests the evolvement of both CYP450s and ODDs (reviewed in Sponsel & Hedden, 2010). A study conducted in rice suggests the initiation of the C-13 hydroxylation pathway functions as a balancing mechanism that favors GA_1 biosynthesis over the more active GA_4 (Magome et al., 2013). Higher GA_4 activity is presumably a result of a greater binding efficiency for the GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), relative to competing 13-hydroxylated GAs (Nakajima et al., 2006; Ueguchi-Tanaka et

al., 2005). As such, 13-hydroxylase enzymes may fine-tune GA-dependent growth responses by tightly regulating bioactive GA levels.

The later steps of GA biosynthesis are catalyzed by cytoplasmic 2-ODDs, GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox). GA20ox is responsible for the sequential removal of C-20 and the formation of the γ-lactone on C-19 and C-10, characteristic of C-19 GAs. In parallel, GA20ox, converts GA_{12} and GA_{53} (13-OH GA_{12}) to GA_9 and GA_{20}, respectively. GA_9 and GA_{20} undergo 3β-hydroxylation, catalyzed by GA3ox, which yields biologically active products, GA_4 and GA_1 (Hedden & Phillips, 2000). While the dominant GA3ox in *Arabidopsis*, *AtGA3ox1*, exhibits high regiospecificity (producing a single product), GA3ox enzymes in monocots possess additional catalytic activity (Sponsel & Hedden, 2010). For example, sequential oxidations of GA_{20} at both the C-2 and C-3 positions, results in the formation of GA_5, which is then converted to GA_3 by oxidation of C-1 and C-3, all by the same enzyme (Itoh et al., 2001).

GA deactivation is also a function of deoxygenase activity. GA2-oxidase (GA2ox) is responsible for catalyzing the irreversible inactivation of GAs by 2β-hydroxylation. In several species, GA2ox enzymes are sub-divided into two classes based on their substrate; a larger class of C-19 GA2oxs and a smaller class of C-20 GA2oxs (Chen et al., 2016; Ci et al., 2021; Honi et al., 2020; Lo et al., 2008; Schomburg et al., 2003). C-19 GA2ox enzymes hydroxylate active C_{19}-GAs, (GA_1 and GA_4) and their C-19 precursors, rendering them inactive. Conversely, C-20-GA2ox enzymes hydroxylate inactive C_{20} precursors (C_{12} and C_{53}), inhibiting their conversion to active forms and thereby reducing the concentration of bioactive hormone (Lo et al., 2008). Alternative forms of GA inactivation include CYP450-mediated epoxidation (Ishida et al., 2022; Zhu et al., 2006), GA conjugation (Schneider et al., 1992), and methylation via GA methyltransferases (GAMT) (Varbanova et al., 2007).

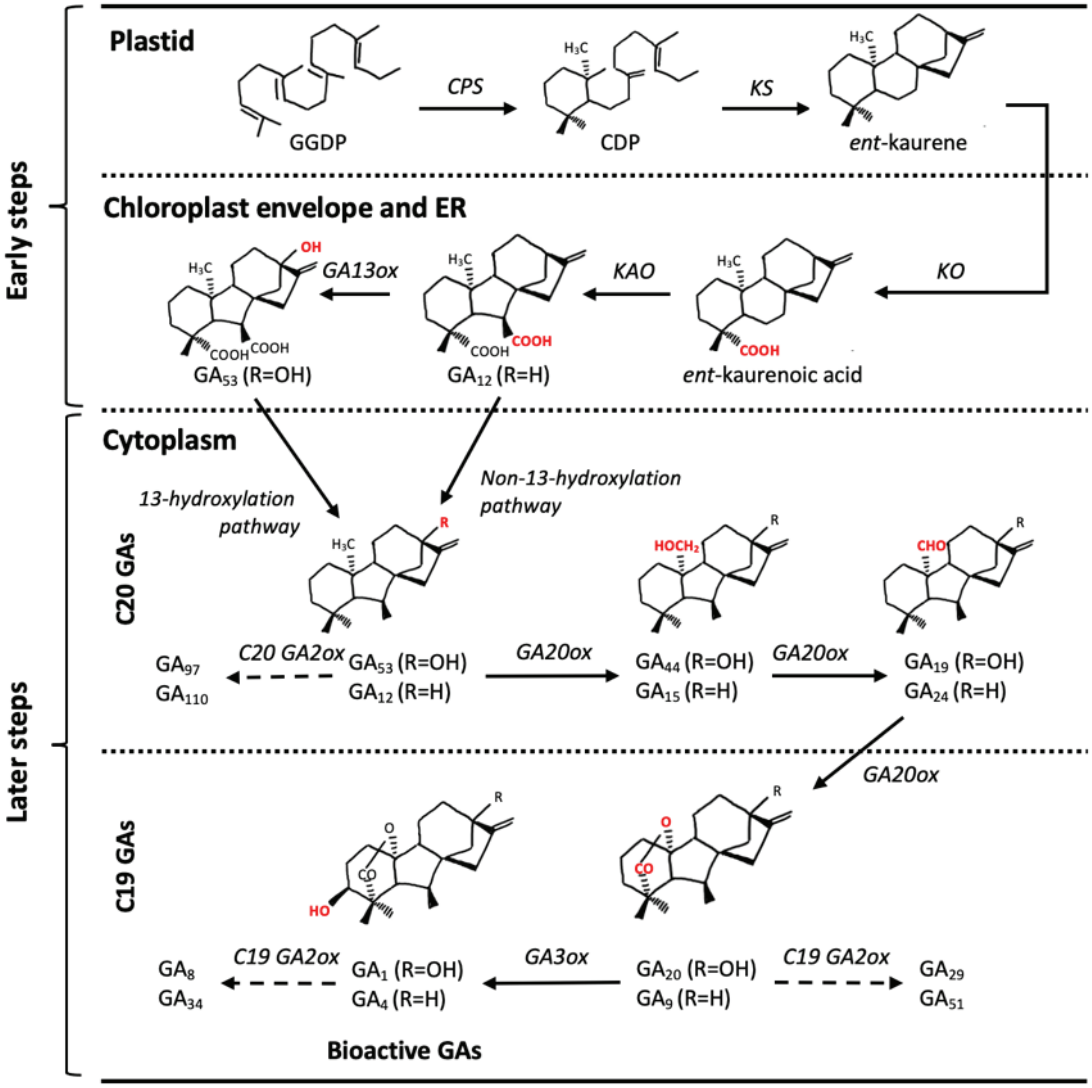

Figure 1. Simplified gibberellin biosynthesis pathway.

The pathway is divided based on localization of metabolites in the chloroplast, endoplasmic reticulum (ER) and the cytosol. The early steps of biosynthesis are catalyzed by terpene synthases *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) and cytochrome P450 monooxygenases (CYP450s) *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). Biosynthesis splits into two parallel pathways and GA_{12} can either undergo C-20 oxidation, or C-13 hydroxylation to produce the precursor GA_{53}. The later steps of the pathway are catalyzed by 2-oxoglutarate, iron-dependent dioxygenases, GA20ox, GA3ox and GA2ox. GA20ox catalyzes multiple steps from GA_{12} to GA_9 and GA_{53} to GA_{20}. GA3ox yields biologically active GA products via 3β-hydroxylation, while GA2ox catalyzes the inactivation of GAs by 2β-hydroxylation. Solid and dotted arrows represent biosynthesis and deactivation steps, respectively.

1.3.3. Gibberellin biosynthesis genes

Arabidopsis mutants, representing lesions at each step of the GA biosynthesis pathway, have helped elucidate gene-function relationships of participating enzymes. Differences in severity of the GA-deficient, dwarf phenotype, correlates with the size of the gene family that encodes each enzyme. In *Arabidopsis,* CPS, KS and KO are encoded by single copy genes, *GA1*, *GA2*, and *GA3* respectively. Null mutations in either gene results in severe dwarfism and infertility, which can be overcome following spraying with endogenous GA (Helliwell et al., 1999; Olszewski et al., 2002; Silverstone et al., 1997). Interestingly, *ga1*, *ga2*, and *ga3* mutants do accumulate some GA, implying that CPS and KS precursors may be synthesized in minute amounts by a related diterpene synthase (Hedden & Phillips, 2000). KAO is encoded by two genes, designated *KAO1* and *KAO2*, which are functionally redundant during seed germination and the development of young organs. Single mutants are indistinguishable from wildtype plants, while the *kao1* and *kao2* double mutant exhibits the typical dwarf, sterile phenotype observed in GA-deficient *ga1-3* (Regnault et al., 2014).

The 2-ODD enzymes are encoded by small gene families with both distinct and overlapping patterns of expression. As such, single-gene loss-of-function mutations produce moderate phenotypes. Plants with defective *ga20ox* or *ga3ox* genes exhibit semi-dwarfism with normal flower development, while overexpression of *GA2ox* produces the dwarf phenotype (Hedden & Phillips, 2000). GA oxidase genes have been characterized in several important crops including; maize (Ci et al., 2021), banana tree (J. Chen et al., 2016), jute (Honi et al., 2020), pea (Lester et al., 1997), barley (Spielmeyer et al., 2004) and grape (He et al., 2019). However, most of our knowledge has come from studies conducted in *Arabidopsis* and rice.

Previous molecular analyses have shown that *Arabidopsis contains five GA20ox*, four *GA3ox*, and eight *GA2ox* genes (Han & Zhu, 2011). In this species and others, the oxidase genes exhibit unique and redundant functions. *AtGA20ox1* and *AtGA20ox2* function redundantly to promote hypocotyl and internode elongation, silique extension, seed development and flower initiation. While *AtGA20ox1* is the dominant transcript in elongating tissues such as the stem, *AtGA20ox2* makes a greater contribution to silique growth and flowering time (Rieu et al., 2008). As such, it is of little surprise that mutations in *AtGA20ox1* produces the semi-dwarf phenotype without pleiotropic effects.

Similarly, *AtGA3ox1* also results in semi-dwarfism, and is responsible for bioactive GA synthesis during vegetative growth and reproductive development (Mitchum et al., 2006). The *ga3ox1* mutant exhibits normal flower development, which appears to be a consequence of contributing *GA3ox3* and *GA3ox4* expression (Hu et al., 2008). Mirroring results have been accomplished in rice (Sakamoto et al., 2004).

Expression of the GA dioxygenase genes is regulated by a number of intrinsic and environmental cues. Several lines of evidence have shown that *GA20ox* and *GA3ox* genes are downregulated by GA, while *GA2ox* is upregulated by GA presence, demonstrating feedback and feedforward regulation (Carrera et al., 1999; Elliott et al., 2001; Honi et al., 2020; Phillips et al., 1995; Toyomasu et al., 1997). In *Arabidopsis*, environmental cues such as red-light exposure and cold stress have shown to increase *AtGA3ox1* transcripts, while repressing *AtGA2ox* expression in imbibed seeds, which requires high endogenous GA for germination (Yamauchi et al., 2004). GA metabolism also appears to be regulated by other hormones. In *Pisum sativum* (pea), removal of the apical bud (a strong auxin source) resulted in a reduction of internodal bioactive GA1, an effect that was reversed following indole-3-acetic acid (IAA) treatment (Ross et al., 2000). In support of this finding, it was later shown that exogenous application of IAA specifically induces the expression of *PsGA3ox1* (Mendel's *Lee* gene), while reducing *PsGA2ox1* transcripts (O'Neill & Ross, 2002). Similar findings have been reported in both tobacco (Wolbang & Ross, 2001) and barley (Wolbang et al., 2004).

1.3.4. High-yield semi dwarfism

The development of high yielding, semi-dwarf varieties in rice and wheat was key to the success of the Green Revolution. Nitrogen-based fertilizer use in the 1960s allowed higher planting density but also promoted an increase in plant stature in high yielding varieties, which consequently could no longer support the weight of heavy grain, and resulted in an increased incidence of lodging (stem collapse) (Hedden, 2003). In contrast, semi-dwarf plants with short, thick stalks, maintained stability with high grain productivity and allowed for an increase use in fertilizer. Molecular studies have revealed that alleles responsible for these traits interfere with the biosynthesis or signal transduction of GA (Hedden, 2003; Peng et al., 1999; Spielmeyer et al., 2002).

The rice dwarfing allele *semi-dwarf-1* (*sd1*) was originally identified from the Chinese cultivar Dee-geo-woo-gen (DGWG). Intensive breeding programs conducted by both Taiwan and the International Rice Research Institute (IRRI), developed the first semi-dwarf rice cultivars, Taichung Native 1 (TN-1) and IR-8 (miracle rice), by introducing the DGWG *sd1* allele into elite varieties. Both cultivars were subsequently used as parents for hybridization to generate many of the commercial varieties grown today. Simultaneous gene characterization by three separate research groups show *SD1* encodes the GA20ox isozyme, *OsGA20ox2*, which normally catalyzes the conversion of the GA precursor, GA_{53} to GA_{20}, ultimately increasing bioactive GA content (Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002). Rice varieties that possess the DGWG *sd1* allele, have a 383-bp deletion that introduces a stop codon and renders the protein inactive (Sasaki et al., 2002). *Sd1* mutants accumulate GA_{53} in their internodes, while products GA_{20} and bioactive GA_1 are reduced relative to tall varieties (Spielmeyer et al., 2002). As *OsGA20ox2* is primarily expressed during vegetative growth, loss-of-function reduces stature without causing detrimental reproductive phenotypes. To date, as many as seven alleles of the *SD1* gene have been identified in rice and used for crop improvement, each of which contain SNPs or insertion-deletions that have arisen spontaneously or by induced mutation (Bhuvaneswari et al., 2020).

As wheat (*Triticum aestivum*) is hexaploid (6n), recessive and null mutations are rarely observed phenotypically due to compensation of similar functioning alleles amongst the other genomes. As such, dwarfing alleles identified in wheat fail to include loss-of-function GA biosynthesis genes. Instead, gene characterization of high yield, semi-dwarf wheat cultivars were shown to be insensitive to bioactive GA. Mutant alleles at the *Reduced height* (*Rht*) locus interfere with GA signal transduction by removing the repressive effects of DELLA proteins (Peng et al., 1999). DELLAs are a sub-group of GRAS transcription factors that suppress GA-responsive genes (Locascio et al., 2013). In wild-type plants, the presence of GA stimulates the formation of the GA-GID1-DELLA complex, which initiates DELLA degradation via the 26S proteosome pathway and consequently the activation of GA-dependent processes. Mutant alleles *Rht- B1* and *Rht-D1*, from the dwarf Norin10, encode defective *N*-terminus DELLA domains (Peng et al., 1999). Loss of the DELLA domain results in reduced affinity for the GID1 receptor, thus, increasing the stability of the protein by limiting its degradation. This alteration in function reduces GA signal transduction and plant height. The independent effects of

Rht- B1 and *Rht-D1* on height reduction are similar, while their combined effects are additive (Hedden, 2003). Both, *Rht-B1* and *Rht-D1* are employed in more than 70% of the world's commercial wheat crop (Hedden, 2003).

The significance of *sd1* and *Rht* phenotypes prompted a push towards ortholog characterization in several economically important crops. Semi-dwarf varieties with deficient GA biosynthesis or signaling, have been successfully developed in barley (Xu et al., 2017), pea (Ross et al., 1989), maize (Lawit et al., 2010), rapeseed (C. Liu, Wang, et al., 2010), banana (Shao et al., 2020), coconut (Boonkaew et al., 2018) and grape (Zhong & Yang, 2012). Although mutation in many genes other than those in GA-related processes can result in semi-dwarfism, few have been utilized for widespread breeding programs. In fact, dwarfism is one of the most common phenotypes in mutant screens (Jürgens et al., 1991), presumably because partial loss of function in many essential genes reduces overall growth, fitness and yield. As such, GA metabolism and signaling genes remains a superior target for alteration of plant stature (Gao & Chu, 2020).

1.4. Hypotheses and intended objectives

The brewing industry continues to rely on the production of new hop cultivars with unique characteristics and flavoring profiles. As hop plants grow unnecessarily tall, a key trait of interest is semi-dwarfism (Darby, 2007; Neve, 2012; Turner et al., 2011). Hop plants require tall trellises to support the growth of their bines, and specialized harvesting equipment is generally required to harvest mature cones, increasing the cost of hop farming. Lower trellises would reduce the cost of harvesting and training as well as provide a better target for spraying against pests and disease. Furthermore, as semi-dwarf plants invest less in internode elongation, a greater proportion of nutrients and photosynthates are partitioned to flower development, resulting in an increase in yield. Despite the obvious need for a semi-dwarf hop cultivar, there has yet to be a successful dwarf brought to market due to poor performance (Neve, 2012; *USA Hops - Hop Growers of America*, *2021*). Moreover, quality characteristics of a given cultivar need to be maintained for brewer acceptance, yet genetic crosses are hindered by the lack of male availability for specific genotypes (Turner et al., 2011).

Recent advances in genome editing known as CRISPR/Cas9, allows us to make programmable and heritable changes to a given genome with little or no off-target

effects. CRISPR requires both a Cas9 endonuclease enzyme capable of cutting double-stranded DNA (dsDNA) and a guide RNA (gRNA), which contains a spacer sequence homologous to the target gene. Following transient or stable expression of the two-component system, the gRNA complexes with Cas9 and guides the enzyme to the complementary target sequence, where upon binding catalyzes a double-stranded break. As repair machinery can be faulty, the rejoining of ends often results in either a deletion or insertion, resulting in a disruption of the genes open reading frame and ultimately loss-of-function. Disruption often occurs in both gene copies of a diploid genome, allowing for immediate recovery of homozygous mutants.

Ideal targets for CRISPR-based development of a semi-dwarf hop are the GA20ox and GA3ox enzymes involved in the later steps of GA biosynthesis. Mutations in *Arabidopsis GA20ox* and *GA3ox* results in a semi-dwarf phenotype with normal flower and seed development when single genes are knocked out (Sponsel & Hedden, 2010). As of recently, two draft hop genomes have been made available (NCBI accessions: PRJNA562558 and PRJDB3233), allowing for the identification of hop *GA20ox* and *GA3ox* orthologs and the synthesis of target-specific gRNAs for CRISPR-induced mutagenesis. As changes in GA content can have both positive and negative effects on flower development, tissue-specific expression of hop GA-oxidase candidates is a required initial step towards engineering a semi-dwarf phenotype with few pleiotropic effects. Ideal gene candidates should have high expression in internodes and low expression in female cones.

A key prerequisite to genetic transformation, is an efficient system for regenerating transgenic plants. Efficiency is dependent on medium composition, hormonal content, explant source and genotype. Previous reports show that hop is highly recalcitrant to *in vitro* shoot regeneration, which is evident by low induction frequencies (Batista et al., 2008; Gurriarán et al., 1999; Horlemann et al., 2003; Okada et al., 2003; Schwekendiek et al., 2005; Škof & Luthar, 2005; Sousa et al., 1995). Moreover, existing published procedures involve a limited number of cultivars. Consequently, new protocols need to be identified for alternative genotypes and optimization of existing methods is required. We have discussed three approaches towards improving shoot regeneration output: optimization of hormone use, chemical enhancement, and the overexpression of key developmental genes. To the best of our knowledge only hormonal induction, largely through trial and error, has been

accomplished in hop. Improving hop regenerative competence will alleviate the bottleneck discouraging genetic transformation and open opportunities for introducing traits ideal for cultivation.

Here we establish the foundation for developing a novel high-yield semi-dwarf hop cultivar, by optimizing shoot regenerative responses and by the identification and characterization of the GA oxidase gene family in the hop genome.

This research is based on the following hypotheses:

I. Hormone-based shoot regeneration in hop can be enhanced through the use of chemical additives and by the transient ectopic expression of embryo and meristem-specific transcription factors. We evaluated shoot-promoting hormone ratios from published literature in two commercially relevant genotypes, Nugget and Galena. Following establishment of a hormone-based regime, we determined if the surfactant PF-68 can improve regeneration competence. Alternatively, we also assessed whether the positive influence of *Arabidopsis thaliana* transcription factors BABYBOOM (BBM) and WUSCHEL (WUS) can enhance transformation output.

II. GA biosynthesis is a suitable target for genetic manipulation towards a semi-dwarf phenotype with normal flower and seed development in hop. To this effect, we identified putative GA metabolism genes in the hop genome and characterized the tissue-specific expression of the *GA20ox* and *GA3ox* gene candidates. Based on our analysis, we synthesized a set of binary plasmids that contain CRISPR expression cassettes designed to knockout two *GA20ox* target genes expressed during internode elongation.

Chapter 2. Methods

2.1. Plant propagation and growth conditions

H. lupulus genotypes, Galena and Nugget, were obtained from Willow Biosciences (Vancouver, B.C.) and Art Knapp Garden Centre (Surrey, B.C.), respectively. To propagate working material, nodal cuttings of each genotype were dipped in Prom-Mix Stim-Root (0.1% IBA) and rooted in hydrophilic propagation rockwool at 25°C, 80% humidity and a 16h/8h photoperiod. Plantlets were watered 1x per week with 1g/L 20-20-20 (NPK). Following six weeks of growth, plants were transferred to 5.5" pots with a peat/perlite mix and placed in greenhouse conditions. To establish *in vitro* cultures, nodal explants were collected from propagated material and surface sterilized in a 70% ethanol wash for 5 min, followed by 7% commercial bleach and 0.01% tween for 10 min. Sterile explants were washed in sterile water (5x) to remove any residual bleach. The basal end of each explant was removed to expose fresh tissue, and explants were transferred to sterile glass culture jars containing plant propagation media (PPM): 1x Murashige and Skoog (MS) (Skoog & Miller, 1957) supplemented with 0.1% MES (PhytoTech, M5531), 2% dextrose (Caledon, 3260-1) and 0.7% plant agar (PhytoTech, A296) at pH 5.8. Cultures were grown under ambient conditions at 22°C under a 16h/8h photoperiod. Growing shoots were excised every 4-6 weeks and sub-cultured onto fresh media. Cultures were maintained for no more than 12 months. Beyond this, a decline in regenerative responses was observed (data not shown).

2.2. Tissue culture assays

2.2.1. Shoot regeneration

Shoot regeneration response was evaluated in genotypes Galena and Nugget by testing various hormone combinations that have shown success in hop. As shoot regeneration has already been achieved in Nugget (Gurriarán et al., 1999), we included this genotype as a comparative measure to identify genotype-specific differences. Hormone supplementation of PPM included IAA, TDZ, zeatin and BAP (Table A.2., Regeneration Media 1, R1a-d). Callus, shoot, and root induction was evaluated in internode, petiole and leaf explants obtained from both greenhouse and *in vitro* material

(6-8 weeks old) (Figure 2A). All explants (5-10 mm) were sampled from the first five nodal segments starting from the tip of the shoot. Leaf explants had their midvein wounded with transverse incisions and were plated abaxial side down. Cultures were grown under ambient conditions at 22°C under a 16h/8h photoperiod.

To identify a shoot multiplication media following regeneration, the effects of various cytokinins were evaluated in nodal culture. Nodes from the genotypes Galena and Nugget were exposed to 5μM TDZ, zeatin or BAP in combination with 1.4μM IAA (Table A.2, Regeneration Media 2, R2a-c). The third to fifth node from the apex was sampled from *in vitro* culture and placed on the surface of the growth media. Ten explants were plated per plate: n= 30 per treatment, per trial. Three independent trials were completed. Cultures were grown under ambient conditions at 22°C under a 16h/8h photoperiod. Shoot number, shoot elongation and node number were assessed following a 4-week incubation. Shoot lengths were measured with an electronic caliper.

When single shoots reached a length of 3-4 cm, they were excised from the original explant and rooted on 0.5 μM IBA (Regeneration Media 3, R3). When removing rooted plants from *in vitro* culture, roots were rinsed in sterile water, and potted in a peat/perlite mix. Plants were placed under domes to maintain high humidity, under indirect light and a 16h/8h photoperiod until acclimated to greenhouse conditions. A schematic representation of the final shoot regeneration procedure is outlined in Figure 5. All media used in culture are summarized in Table A.2.

2.2.2. Pluronic-F68 exposure

In vitro internodes derived from the genotype Galena were sampled as previously described and plated on R1a media supplemented with increasing concentrations of commercial grade Pluronic F-68 (PF-68): 0%, 0.001%, 0.01%, and 0.1%. Shoot initiation was scored following a six-week incubation period. Ten percent (w/v) stock solutions were prepared by dissolving 1g of PF-68 (Sigma, #P7061) into 10 ml of distilled water, followed by a brief heating to 40-50°C and filter sterilization.

2.2.3. Hygromycin dose-response

The effect of Hygromycin B (PhytoTech, #H397) on non-transformed genotypes Galena and Nugget as well as *Hpt* positive, *AtBBM* shoots (Galena background) were tested in nodal culture. The third to fifth node was sampled from in vitro culture and

plated directly on the surface of R2c media supplemented with increasing concentrations of hygromycin: 0, 0.75, 1.5, 2.5 and 5 mg/L. Ten explants were plated per plate: n= 30 per treatment, per trial. Three independent trials were completed. Tissue was maintained on selection media for a period of six weeks. Cultures were grown under ambient conditions at 22°C under a 16h/8h photoperiod. Shoot length, rooting and tissue death was evaluated.

2.3. GA_3 and paclobutrazol treatment

Cuttings from the genotype Galena were dipped in PROMIX STIM-ROOT and rooted in rockwool for 7-days. Rooted cuttings with shoots of near equal lengths were selected and organized at random for weekly watering treatments of exogenous GA3 (Gro Spurt, #GS5) or paclobutrazol (PB, Sigma, #43900). Initial shoot lengths from both axial buds were measured from the primary node of each cutting prior to treatment. Plantlets were watered with 1g/L 20-20-20 (NPK), supplemented with either 0, 2.5 or 5 ppm of either GA3 or PB once a week. Following 30 days of exposure, the dominant shoot of each plant was measured from the primary node, and initial shoot lengths were subtracted to determine shoot growth. Two independent growth trials were completed with a total sample size n= 18-38 plants per treatment.

2.4. Gene identification and phylogenetic analyses

2.4.1. GA biosynthesis gene identification

CDS and protein sequences that encode the key GA biosynthesis genes were retrieved from TAIR (The *Arabidopsis* Information Resource, http://www.Arabidopsis.org) and NCBI (http://www. ncbi.nlm.nih.gov/). To identify homologs in the hop genome, we conducted a basic local alignment search using known sequences in *Arabidopsis* and *Cannabis*, against *H. lupulus var. lupulus,* genotype Cascade (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA562558/). Predicted open reading frames were translated to amino acid sequences using the translate tool available on Expasy (https://web.expasy.org/translate/) and subcellular protein localization was predicted by Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). Lastly, domain signatures were identified by Interpro (https://www.ebi.ac.uk/interpro/).

2.4.2. Gene identification and phylogenetic relationships

Protein sequences of *Arabidopsis*, *Cannabis*, and *H. lupulus* were aligned with MUltiple Sequence Comparison by Log-Expectation (Muscle) and phylogenetic trees were constructed in Geneious (Biomatters, Ltd., Auckland, New Zealand) using the UPGMA algorithm. Bootstrap values were obtained using 1000 replicates. Conserved motifs were identified by Multiple Expectation Maximization for Motif Elicitation (MEME) (http:// meme-suite.org/). Parameters were set to identify a maximum of 15 ungapped motifs, with a maximum motif length of 6-50 amino acids.

2.4.3. Primer design

qRT-PCR Primers specific to *GA20ox* and *GA3ox* sequences were identified for the *H. lupulus* genotype Cascade using the NCBI Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). A summary of all primers used in our experiments can be found in Table A.3. Parameters were set to a product length of 70-200-bps and a melting temperature (T_m) of 63°C, with a max Tm difference of 2°C between primers. Melting curves of the PCR products from the samples tested indicated that each primer pair generated specific PCR amplifications. In addition, single products were confirmed through regular PCR and gel electrophoresis.

2.4.4. Tissue-specific expression

RNA was extracted from apical and basal internodes, immature and mature leaves, roots and cones using the CTAB method (Rajakani et al., 2013). Apical and immature leaves were sampled from the apical shoot tip and basal internodes and mature leaves were sampled from the oldest node of the primary branch. All explants were taken from 6-month-old plants except roots, which were excised from 4-week-old rooted cuttings. Relative expression was measured with BrightGreen 2x qPCR MasterMix by ABM (MasterMix-S-XL). The total reaction volume was 20µl, consisting of 3µl of dilute cDNA template. Thermocycler parameters were set to 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Expression levels were measured using the 2^-($\Delta\Delta C_t$) method. Gene expression was normalized using average C_t values obtained from the reference genes *7sl-RNA* and *GAPDH.* Both have shown to be appropriate standards for qRT-PCR use in hop (Maloukh et al., 2009). The tissue with

the lowest expression value (highest average C_t) functioned as a control for each analysis. Obtained C_t values are an average of three technical replicates per biological sample. Technical replicates had an acceptable error of <0.3 C_t. Three biological replicates were included.

Tissue-specific expression was evaluated for each *GA20ox* and *GA3ox* gene individually, as well as across each sub-family. To identify *GAox* targets for CRISPR-induced deletions towards a semi-dwarf phenotype with normal flower development, *GA20ox* and *GA3ox* genes were ranked based on a preference of high expression level in apical internodes and low expression level in developing female cones.

2.5. Vector synthesis and cloning

2.5.1. guide RNA design

Single guide RNAs (gRNA) specific to *H. lupulus GA20ox* candidates, *HlGA20ox1* and *HlGA20ox2,* were designed with the online tool CRISPR RGEN (http://www.rgenome.net/) using the available reference genome for the *H. lupulus* genotype Shinsuwase (PRJDB3233). Target sites (also referred to as the gRNA spacer) were located immediately upstream the protospacer adjacent motif (PAM), NGG, specific to *Streptococcus pyogenes* Cas9. Each spacer was selected based on a 50-80% GC content and an out-of-frame score greater than 66 (with the exception of *HlGA20ox2*, gRNA2). Both the Shinsuwase and Cascade genomes were screened for potential off-target effects. Sequences with off-target sites that contained <3 miss-matches were avoided. Spacers were commercially synthesized to contain 5'-adapter sequences that complement the *Bsa*I restriction site (Table A.3). Complementary oligos were annealed to form oligoduplexes using the following reaction: 1 μL (10 pmol) per oligo, 43 μL water and 5 μL 10x annealing buffer (10 mM Tris pH 8, 50 mM NaCl and 1 mM EDTA). Each reaction was placed in a thermocycler at 95°C for 5 min, followed by a ramp down to 25°C at 1°C per min.

2.5.2. gRNA cloning

The plant-specific CRISPR vector *pHSE401* (Addgene, U.S.A., Plasmid #62201) was cut with the *Bsa*I-HF restriction enzyme (NEB, #R3535) and column purified to remove the 1219-bp Spectinomycin resistance gene (SmR) fragment. Single

oligoduplexes with 5' overhangs were introduced into the digested *pHSE401* vector by a standard T4 ligase sticky-end ligation reaction. Annealed vectors were cloned in DH5α, 10^8 cells (MacCell^{TM}, #15053) following provided procedure and then purified using a GeneJET Plasmid Miniprep Kit (ThermoFisher, #K0503). Cloning of gRNA fragment was confirmed by colony PCR using primers *U6-F* and *U6-R* (Table A.3) that flank the gRNA cloning site (Xing et al., 2014). An expected band size of 423-bp suggested introduction of the correct product. As cloning of artifacts can occur, PCR positive vectors were sequenced by Sanger sequencing using the *U6-F* primer.

2.5.3. Sequence and ligase independent cloning of 35S::AtBBM:GR

Sequence and ligase independent cloning (SLIC) was adapted from a published protocol (Li & Elledge, 2007). *pHSE401* vectors containing spacer sequences specific to *HIGA20ox1* and *HIGA20ox2* were cleaved with FastDigest *EcoR*I (ThermoFisher, #FD0275). The 3292-bp *35S::AtBBM:GR* expression cassette was copied from the *pH35S.BBM* overexpression plasmid (Willow Biosciences, Vancouver, B.C.) by PCR with forward and reverse primers that included 15-bp's of homology to the flanks of the *EcoR*I cut site (Table A.3). Both the PCR insert, and linearized vectors were column purified and then independently treated with T4 DNA polymerase (NEB, #M0203S) in T4 buffer plus BSA for 30 min at room temperature. T4 DNA polymerase has exonuclease activity that results in the production of complementary overhangs in both the vector and insert. Reactions were stopped by the addition of dCTP. A 1:1 insert to vector molar ratio was added to 10x T4 ligation buffer (NEB, #B0202S) and incubated for 30 min at 37°C. 3 μL of each annealing reaction were independently added to 50 μL of DH5α, 10^8 cells (MacCell^{TM}, #15053) following the provided procedure for transformation via heat-shock and then plated on solid Luria-Bertani (LB) media supplemented with 50 mg/L kanamycin. Plates were incubated at 37°C for 16 hours. Isolated colonies were chosen at random for colony-PCR using *AtBBM*-specific primers, *BBM_F* and *BBM_R* (Table A.3). Plasmid DNA from *AtBBM* positive colonies was amplified by PCR, purified and the ligation of the *35S::AtBBM:GR* expression cassette (35S promoter to the tNos terminator) was confirmed by Sanger sequencing using primers *SLICseq_F* and *M13_R* (Table A.3).

2.6. Genetic transformation

2.6.1. Agrobacterium preparation

All plant transformations were mediated by *Agrobacterium tumefaciens.* Expression vectors *pMDC164_35SGUS, pH35S.BBM* and *pH35S.WUS* were acquired from Willow Biosciences (Vancouver, B.C.), while *pHSE401* was obtained from Addgene, as previously stated. Each vector (obtained or modified) were electroporated into the *Agrobacterium* strain EHA105 following published procedure (Weigel & Glazebrook, 2006). Single colonies were PCR screened for plasmid uptake by detection of the T-DNA specific-marker *Hpt* and for the presence of the virulence plasmid by virulence (*vir*) genes *virE3* and *virD5*.

Prior to transformations, *Agrobacterium* containing the desired binary vector was grown in 3 ml of liquid LB media supplemented with the appropriate selection for 24 hours at 28°C, 200 rpm, in the dark. Afterwards, two plates per transformation were streaked on solid LB and grown for an additional two days under the same temperature and light conditions. On the day of co-culture, each plate was scraped and re-suspended in 100 ml of liquid co-culture media (Co.1) and the optical density at 600 nm adjusted to 0.8 (Logemann et al. 2006).

2.6.2. Co-culture and regeneration of transformed plants

Internodes (~5-10 mm) from *in vitro*-grown Galena plants were excised, placed directly into re-suspended *Agrobacterium* culture, and agitated at 200 rpm in the dark for 30 min. Explants were blotted dry and transferred to fresh plates containing solid co-culture media (Co.2) topped with a single sheet of sterile filter paper to prevent overgrowth of bacteria. Plates were incubated at ambient temperature (22°C) in the dark for 3 days.

Following co-culture, explants were rinsed in sterile water containing 200 mg/L Timentin and plated onto shoot Regeneration Media, R1a (unless otherwise specified in text) with 0.75-1.5 mg/L hygromycin. Following shoot formation, organogenic explants were taken off selection and transferred to R2c media for shoot multiplication. Shoots 3-4 cm in length were excised from original explants and rooted in media R3. All media post-co-culture was supplemented with 200 mg/L Timentin. Timentin exposure was

sufficient at removing *Agrobacterium* when callus/shoots were sub-cultured every three weeks for three months. Media used in culture is summarized in Table A.2.

2.6.3. Genotyping

Gus staining of internodal callus tissue was conducted according to Jefferson (1987). Genomic DNA was extracted using a CTAB-DNA precipitation method, followed by a silica-based DNA clean-up step (Xin & Chen, 2012). Genetic screening of potential transgenic shoots was performed by PCR >3 months post-regeneration. Primers used to confirm genomic integration of T-DNA can be found in Table A.3. Additional tests for evaluating the stability and expression of our transgenes in soil-grown plants is currently being completed.

2.7. Statistical analyses

Statistical analyses were performed in JMP (SAS, Cary, NC, USA) using a one-way analysis of variance (ANOVA) test and Student's t test ($p < 0.05$). Associated graphs were generated in Excel. All experiments were conducted with ≥3 independent trials, unless otherwise stated.

Chapter 3. Results

3.1. Shoot regeneration responses in the Galena and Nugget cultivars

The success of *Agrobacterium*-mediated genetic transformation is limited not only by frequency of stable cell transformation, but also the ability of cells to form shoots *de novo*. Current publications on hop tissue culture show that *de novo* shoot formation from calli occurs at low frequency also in the absence of *Agrobacterium* infection and antibiotic selection (Batista et al., 1996; Gurriarán et al., 1999) In addition, regeneration responses vary considerably between cultivars. We therefore began by testing the responses of two current and regionally popular cultivars, Galena and Nugget, to published shoot induction media, preferred shoot elongation media, and the potential benefit of a tissue culture enhancer.

3.1.1. Hormone-induced shoot regeneration

We evaluated the response of the cultivars Galena and Nugget on different cytokinin/auxin combinations based on published procedure that elicit a shoot regeneration response in hop (Gurriarán et al., 1999; Horlemann et al., 2003; Mishra et al., 2018). Internodes, petioles, and leaf segments (~5-10 mm) were sampled from *in vitro*-grown Galena and Nugget plantlets and assessed for differential responses following a 6-week exposure period on MS-supplemented Regeneration Media (R1) (Table 1).

All hormone combinations evaluated resulted in callus initiation, which was observed growing at the excision sites of all three explant-types. Callus was moist, with a compact center and ranged in color from pale yellow to brownish black. Browning was far more apparent in Nugget-derived callus regardless of explant and hormone exposure. Clusters of green surface calli were also observed in both cultivars, but their presence did not appear to correlate with the initiation of shoots (data not shown).

Regenerated shoots all appeared indirectly following an intermediate callus phase, despite culturing explants directly onto cytokinin-rich SIM. Media supplemented with 9μM TDZ and 1.4μM IAA (R1a) showed the highest shoot-inducing capacity, with 3.5% of Galena-derived internodes initiating shoots (Table 1). Nugget internodes

produced shoots at 0.7% on the same media. In addition, this was the only medium capable of promoting shoot development from all three explant types sampled from Nugget (Figure 2). The second most effective shoot induction media contained 4.6μM zeatin and no auxin (R1c). For comparison, 0.3% of Galena and 0.8% of Nugget internodes initiated shoots, with potentially higher response rates in petioles (Table 1). Media with 8.8μM BAP (R1d) resulted in shoot formation only from Nugget petioles (0.8%).

Surprisingly, both cultivars failed to form shoots on media containing 4.6μM zeatin and 1.4μM IAA (R1b), and despite the high cytokinin to auxin ratio, roots developed from calli or directly from the explants at high frequency. Media supplemented with 4.6μM zeatin and 1.4μM IAA was the most effective cytokinin/auxin ratio for promoting root initiation in all three explant-types (Table 1). Leaf tissue was the best source for root regeneration, with 12.9% of explants from Galena and 9% of explants from Nugget exhibiting rhizogenic responses. BAP-containing media was also efficient at forming roots from leaf explants in both cultivars, though at lower frequencies; 4.6% (Nugget) and 3.3% (Galena). Root formation was absent in all explants in the presence of TDZ.

Recorded frequencies of explants capable of initiating shoots were consistently low and varied from trial to trial. Despite these observations, once shoot development was initiated, several shoots grew in tandem from each explant. A subset of proliferating calli that failed to initiate shoots within the six-week time frame of evaluation were sub-cultured onto fresh media every four weeks and maintained over a six-month period. None of the cultured calli resulted in shoot induction, suggesting callus tissue lacked organogenic capacity past six weeks. Surface-sterilized explants sourced directly from greenhouse material were also evaluated. Despite having more prominent callus growth than *in vitro* tissue, no organ regeneration occurred and calli eventually turned black and died (data not shown).

Table 1. Hormone-induced regeneration responses.

Hormone-induced regeneration responses. Evaluated Regeneration Media (R1) were adopted from: Horlemann et al. 2003 (R1a), Mishra et al. 2018 (R1b), and Gurriarán et al. 1999 (R1c,d),. Shoot and root % = (number of organogenic explants/total number of explants (n)*100). Mean±SE. Means represent the average % of five independent growth trials. Statistical significance was determined using a pair-wise Student's t-test ($p<0.05$). Values not represented by the same letter, were found to be significantly different.

		9μM TDZ + 1.4μM IAA (R1a)			4.6μM Zeatin + 1.4μM IAA (R1b)		
Explant	Genotype	(n)	Shoot %	Root %	(n)	Shoot %	Root %
Internode	Galena	245	3.5 ± 0.7^{d}	0	256	0	4.3 ± 1.2^{d}
	Nugget	239	0.7 ± 0.7^{e}	0	245	0	1.8 ± 1.3^{e}
Petiole	Galena	90	0	0	121	0	6 ± 3.7^{c}
	Nugget	101	0.7 ± 0.7^{e}	0	93	0	0
Leaf	Galena	80	0	0	93	0	12.9 ± 7^{a}
	Nugget	103	0.9 ± 0.9^{e}	0	103	0	9.0 ± 4.3^{b}

		4.6μM Zeatin (R1c)			8.8μM BAP (R1d)		
Explant	Genotype	(n)	Shoot %	Root %	(n)	Shoot %	Root %
Internode	Galena	267	0.3 ± 0.2^{e}	0	265	0	0
	Nugget	233	0.8 ± 0.8^{e}	0	199	0	0
Petiole	Galena	173	1.5 ± 1^{e}	0	178	0	0
	Nugget	144	0.6 ± 0.6^{e}	0	93	0.8 ± 0.8^{e}	0
Leaf	Galena	70	0	1 ± 1^{e}	76	0	3.3 ± 2.0^{d}
	Nugget	76	0	0	61	0	4.6 ± 3.0^{d}

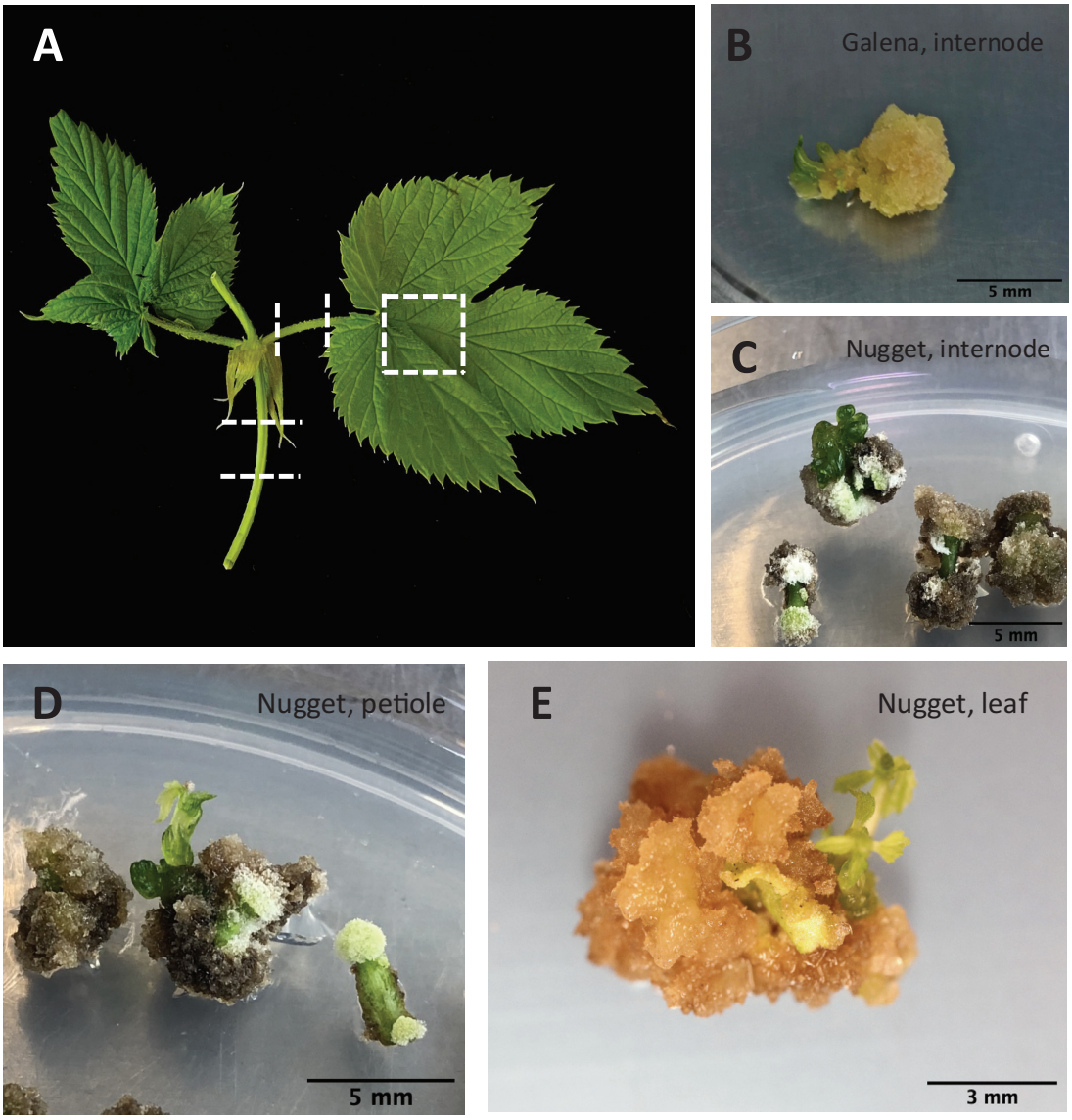

Figure 2. TDZ-induced shoot regeneration.
Explant material for regeneration (A). Regenerated shoots following a 4-6-week exposure to 9μM TDZ and 1.4μM IAA (R1a) from Galena internode tissue (B); Nugget internode tissue (C); Nugget petiole tissue and (D) Nugget leaf tissue (E).

3.1.2. Identification of suitable multiplication medium

Internodes exposed to medium containing TDZ and IAA showed a significantly higher shoot regeneration response than the other tested hormones, specifically in the cultivar Galena (Table 1), making this the ideal combination for additional regeneration

experiments. As morphological defects are often observed in the presence of high TDZ concentrations or prolonged TDZ exposure, initial shoots are generally transferred to an alternative cytokinin source or a lower TDZ concentration. Hence, we evaluated shoot growth from stem nodal explants incubated on different cytokinin sources, TDZ, zeatin and BAP, to identify a shoot multiplication media (Regeneration Media 2, R2) for newly formed shoots. Cytokinins were supplemented at the 5μM level in combination with 1.4μM IAA (Figure 3). Plantlet growth was assessed following a 30-day incubation period.

All three hormone combinations induced a large calli at the base of shoots compared to the no-hormone control (Figure 3.A). Nodes exposed to TDZ developed pale green shoots with swollen apexes and vitrified leaves. We also noted abnormal leaf margins and callus formation at the base of developing leaf primordia. Shoots that emerged in the presence of either BAP or zeatin appeared morphologically normal with thicker internodes and enhanced leaf development. Following a 30-day exposure period, shoot number increased significantly in nodes exposed to BAP compared to TDZ and zeatin. For example, a 33% (p= <0.0001) and 28% (p= 0.0005) difference was observed between BAP and TDZ and a 47% (p= <0.0001) and 42% (p= <0.0001) difference was observed between BAP and zeatin, in Galena and Nugget, respectively (Figure 3.B). On average BAP increased shoot formation by 39% in Galena and 33% in Nugget compared to the no-hormone control (p= <0.0001, <0.0001). Interestingly, zeatin exposed nodes sampled from Galena, resulted in a 23% reduction in shoots formed (p= 0.0095). Although several shoots appeared, we often saw a single dominant shoot in the zeatin cultures (Figure 3.A). No significant differences were observed between zeatin and control cultures in Nugget, which may have been a consequence of poor response to both treatments.

We found a clear correlation between cytokinin treatment and shoot elongation (Figure 3.C). On average, shoots growing on either BAP or zeatin-media were 64% (p= 0.0008) and 57% (p= 0.0095) longer in Galena compared to the no-hormone control. A stronger response was observed in Nugget, where shoots increased in length by 240% (<0.0001) and 230% (<0.0001). Interestingly, no difference in shoot length was found between cultivars in either BAP or zeatin cultures, despite Galena preforming better than Nugget in media without hormone supplementation. Node number per stem also increased significantly in response to zeatin and BAP (Figure 3.D). In contrast, Galena-

derived shoots exposed to TDZ developed fewer nodes with shoot elongation inhibited compared to the control (p= 0.0044), while no significant differences were found in Nugget (Figure 3).

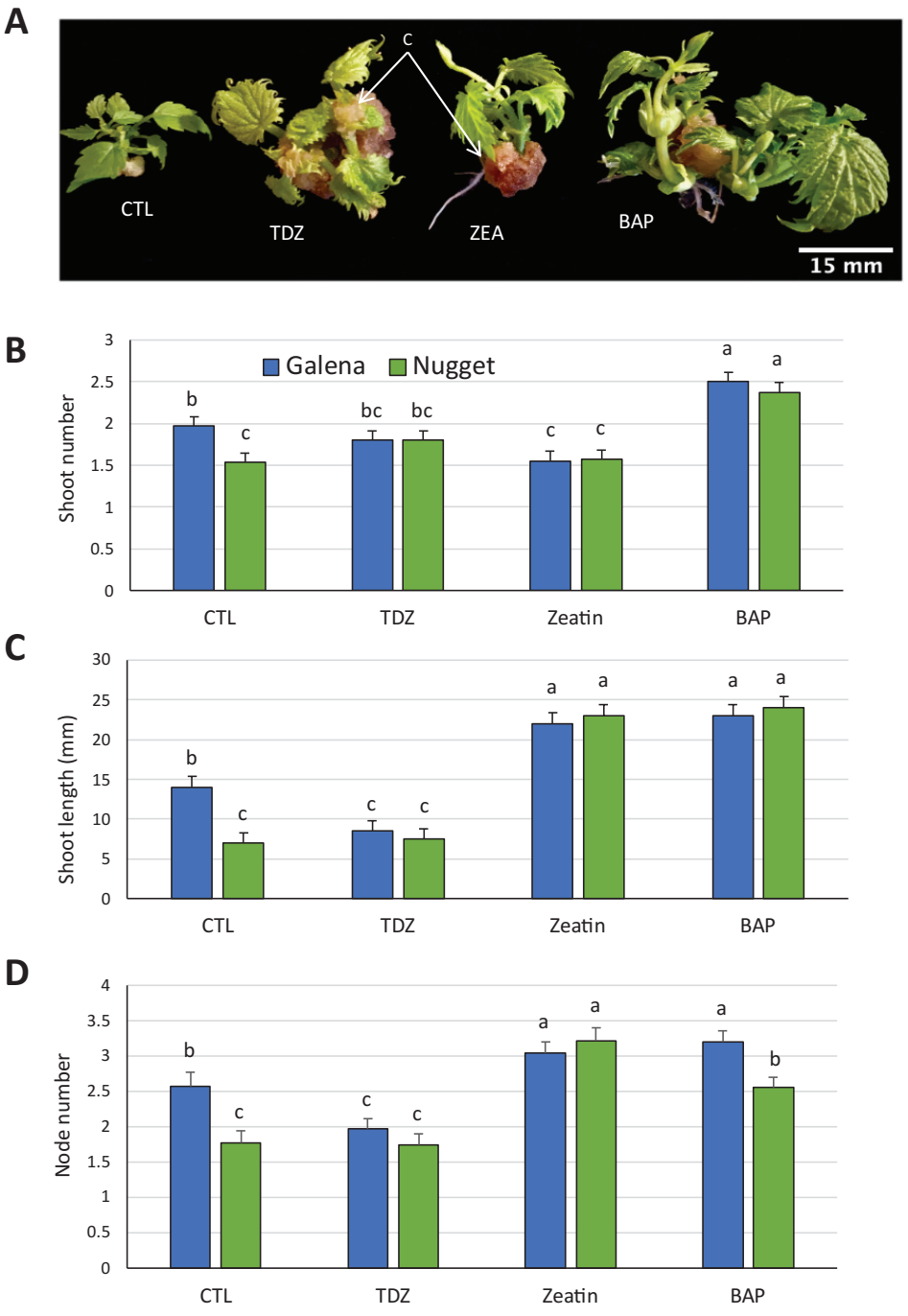

Figure 3. Shoot growth response to cytokinins in nodal cultures.
Shoot morphology was assessed in nodal explants from cultivars Galena and Nugget following exposure to 1.4μM IAA in combination with either 5μM TDZ, zeatin or BAP (Regeneration Media 2, R2a-c). Phenotypes observed in Galena are depicted in image (A). Control, CTL, consists of no hormone background: MS-B5, 2% glucose. Shoot number (B), shoot length (C) and node number (D) were scored following a 4-week exposure period. Arrows identify callus, c, formation. Values were obtained from three independent growth trials, n=30 per treatment, per trial. Statistical significance was determined using a pair-wise Student's t-test (p<0.05). Values not represented by the same letter, were found to be significantly different.

3.1.3. Root induction

The hop plant was relatively simple to root *in vitro*. Shoot multiplication media R2b (zeatin) induced root formation in 96% (Galena) and 93% (Nugget) of nodal cultures (Figure 4). Comparatively, roots developed in 89% (Galena) and 80% (Nugget) of cultures when exposed to BAP. No significant difference was observed between cultivars in either treatment. Only 13% of Galena cultures and 0.3% of Nugget cultures produced roots in the absence of hormones, while no cultures developed roots on TDZ.

In a separate experiment, *in vitro* shoots sampled from the cultivar Galena were excised and placed on plant propagation media (PPM) supplemented with 0.5 μM IBA (Regeneration Media 3, R3). Rooting was successful in 100% of explants (data not shown).

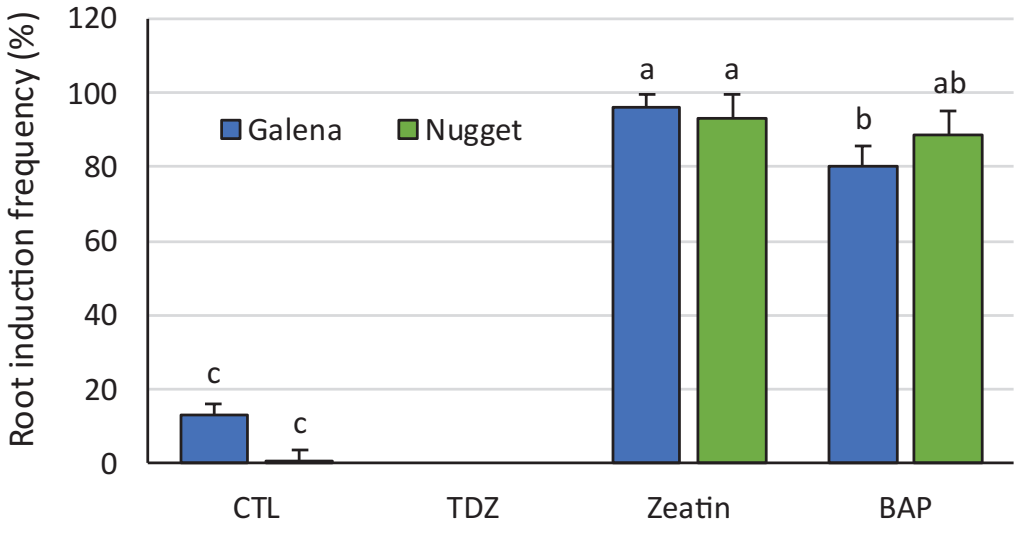

Figure 4. Root induction response to cytokinins in shoot cultures.
Root induction was assessed in nodal explants from cultivars Galena and Nugget following exposure to 1.4μM IAA in combination with either 5μM TDZ, zeatin or BAP (R2a-c). Control, CTL, consists of no hormone background: MS-B5, 2% glucose. Cultures were scored following a 4-week exposure period. Values were obtained from three independent growth trials, n=30 per treatment, per trial. Statistical significance was determined using a pair-wise Student's t-test (p<0.05). Values not represented by the same letter, were found to be significantly different.

3.1.4. Established regeneration system

A schematic representation of the shoot regeneration protocol developed for the cultivar Galena is depicted in Figure 5. Internodes (~5-10 mm in length) were excised from 6-8 week old *in vitro* propagated material and placed on R1a. On average 3.5% of explants developed shoots within four weeks in culture (Table 1). Once shoots were initiated, organogenic tissue was transferred to R2c, which was chosen for its ability to increase shoot number, promote shoot elongation and initiate rooting (Figure 5). Following a 2-week incubation period, elongated shoots from each cluster were separated and placed on fresh R2c media for an additional 2-4 weeks. Shoots growing on R2 generally appeared healthy with dark green vegetation. To promote rooting, excised shoots from shoot clusters were subcultured onto R3. Following root induction, individual plantlets were transferred to soil and placed in indirect light with high humidity. Overall, the regeneration procedure from explant to greenhouse conditions ranged from 4-6 months depending on the viability of the shoots regenerated.

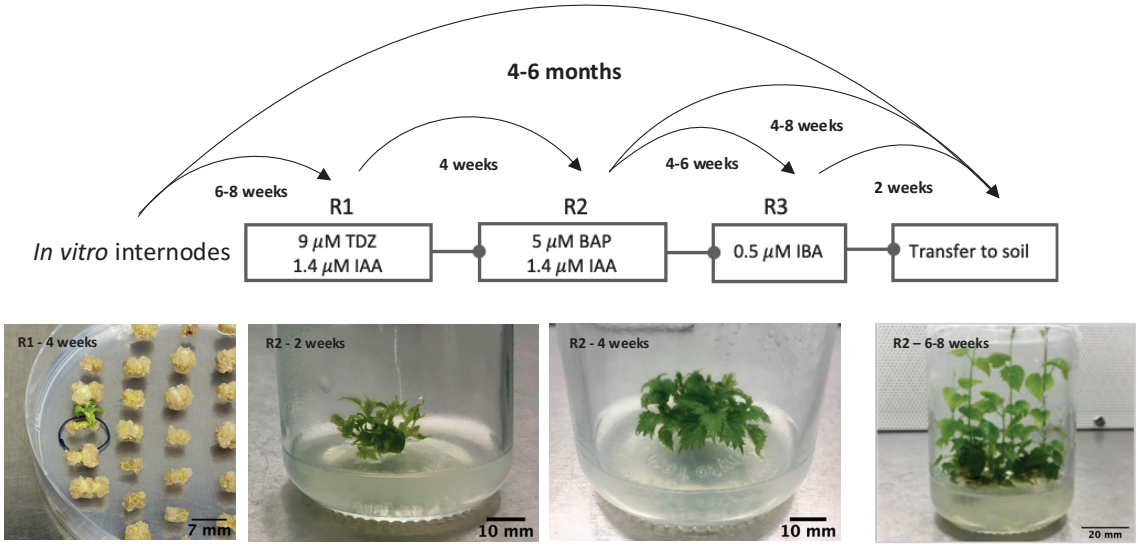

Figure 5. Shoot regeneration protocol established for the cultivar Galena.
Flow diagram including successive hormone treatments and appropriate sub-culturing times with representative images. Regeneration Media 1 (R1) = shoot induction; Regeneration Media 2 (R2) = shoot multiplication; Regeneration Media 3 (R3) = root initiation.

3.1.5. Chemical regeneration enhancers – Pluronic F68

The non-ionic surfactant Pluronic F68 (P-F68) is often used in culture to promote shoot proliferation and enhance organogenesis. To our knowledge there are no published reports using chemical regeneration enhancers in combination with shoot promoting hormones in hop. Therefore, we evaluated shoot regeneration from Galena-derived internodes using R1a media supplemented with three different concentrations of P-F68 (0.001%, 0.01% and 0.1%) (Figure 6.A). Cultures were evaluated following a 6-week incubation period. The frequency of explants developing shoots increased ~3-fold from 0.6% at 0% P-F68 to 2.4% (p= 0.0016) at 0.01% P-F68. Similarly, we saw a ~2.4-fold increase from 0.6% to 2% (p= 0.016) at 0.1% F68. We also found no visual differences in shoot morphology between cultures with and without supplementation, suggesting P-F68 does not appear to negatively affect shoot development within the chosen concentration range. However, callus grown on P-F68 supplemented media exhibit far less tissue browning (Figure 6.B).

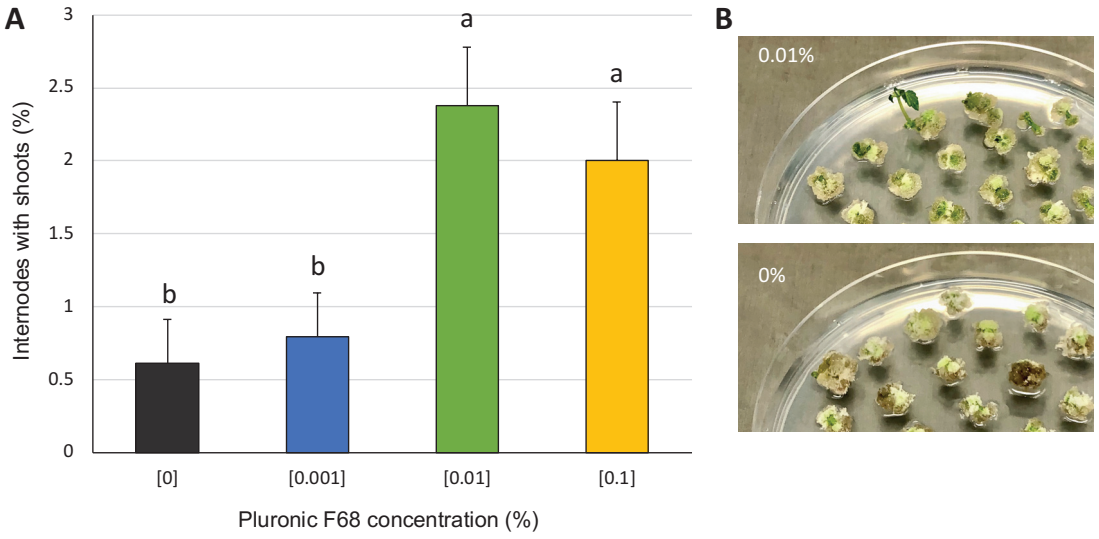

Figure 6. P-F68 exposure enhances hormone-induced shoot regeneration.
Shoot regeneration from Galena-derived internodes using R1a media supplemented with three different concentrations of P-F68 (0.001%, 0.01% and 0.1%) (A) Regeneration frequency was calculated by the number of explants forming shoots/total explant number. Values were obtained from three independent growth trials; n=150-180 explants per concentration, per trial. Statistical significance was determined using a pair-wise Student's t-test (p<0.05). Values not represented by the same letter, were found to be significantly different. Cultures grown in the presence of 0.01% P-F68, visually exhibit less browning than the 0% P-F68 control (B).

3.1.6. BABYBOOM and WUSCHEL-mediated transformation

The overexpression of the transcription factors *BBM* and *WUS* have been shown to induce shoot regeneration in recalcitrant species and improve overall transformation efficiency (Jha et al., 2020; Jha & Kumar, 2018; Lowe et al., 2016). Since constitutive expression of such factors throughout development can lead to pleiotropic phenotypes, the use of inducible expression systems can restrict gene function outside of embryo formation and shoot induction and allow the plant to proceed through normal development (Lutz et al., 2015). To determine whether *Arabidopsis BBM* and *WUS* can mediate shoot regeneration in hop, we obtained two dexamethasone (DEX)-inducible expression plasmids from Willow Biosciences Inc (Vancouver), *pH35S.BBM* and *pH35S.WUS*, that contain the *Arabidopsis* open reading frames of either gene (Figure 7).

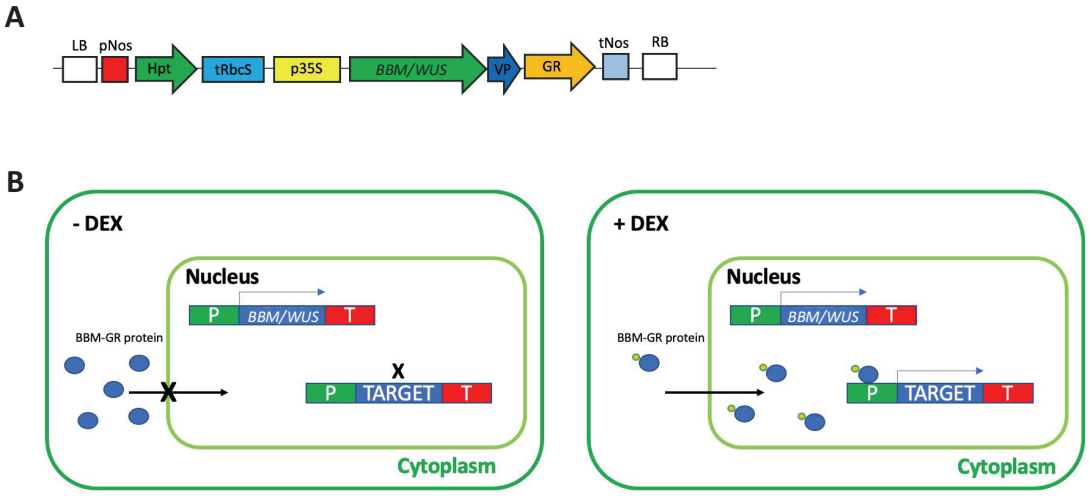

Figure 7. Dexamethasone-inducible system for ectopic expression of transcription factors AtBBM and AtWUS.

T-DNA map of *p35S.BBM* and *p35S.WUS* (A). The expression cassette consists of the hygromycin phosphotransferase (*Hpt*) gene from *E. coli* under the control of the Nopaline synthase promoter (pNos) and Rubisco terminator (tRbcS). *AtBBM* or *AtWUS* expression is driven by a 35S promoter from the cauliflower mosaic virus (CaMV) and the transcriptional activator (VP16). Expression is terminated by the Nopaline synthase terminator. The coding region of either *AtBBM* or *AtWUS* is fused with a glucocorticoid receptor (GR), that recognizes the synthetic steroid dexamethasone (DEX). (B) When plant growth media is supplemented with DEX, DEX binds GR and the transcription factor can localize to the nucleus and activate target genes involved in embryogenesis and meristem establishment. In the absence of DEX, the fusion protein remains in the cytoplasm and is inactive, despite being constitutively expressed (Lutz et al., 2015).

Internodes from the genotype Galena were transformed using *Agrobacterium-mediated* transformation with either *pH35S.BBM* or *pH35S.WUS* expression vectors. To visualize transformation events, *p35S.GUS* transformations were completed as a positive control. Following co-culture, internodes were placed on either hormone-free PPM media with 5 μM DEX, R1a media with 5 μM DEX or R1a media without DEX. All plates were supplemented with 1.5 mg/L Hygromycin B to select for antibiotic resistance in transgenic shoots and 200 mg/L Timentin to inhibit *Agrobacterium* growth post cocultivation.

Cultures were assessed for shoot regeneration following four to six weeks on antibiotic selection (Figure 8, Table 2). No shoots were obtained from our *p35S.GUS* transformations (three trials; n= 343 internodes), though transgenic callus expressing GUS was confirmed (Figure 8.A, Table 2). After transformation with *pH35S.BBM* or *pH35S.WUS, s*hoots appeared to develop both directly from excision sites of internodal explants (Figure 8.B) and indirectly from callus (Figure 8.C). Direct shoot regeneration, generally occurred within two weeks on selection, while indirect shoots developed within three to four. Once shoots were initiated, explants competent of shoot organogenesis were taken off DEX and placed onto fresh media following the regeneration procedure outlined in Figure 5. Lines from both *pH35S.BBM* and *pH35S.WUS* appeared phenotypically normal, suggesting transient expression of either transgene had little impact on development post-regeneration (Figure 8.E,F).

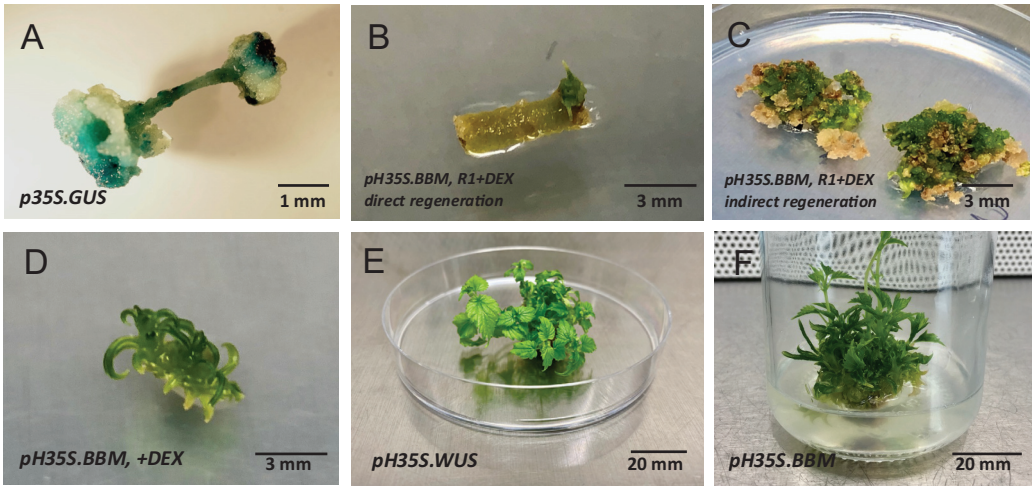

Figure 8. Regenerated shoots from pH35S.BBM and pH35S.WUS responsive explants. Transgenic callus positive for *GUS* (A). Direct regeneration of *pH35S.BBM* positive shoot on R1a media with DEX (B). Indirect regeneration of *pH35S.BBM* induced shoot apical meristem clusters on R1a media with DEX (C). *pH35S.BBM* regenerated shoots on hormone-free PPM media supplemented with DEX (D). *pH35S.BBM* (E) and *pH35S.WUS* (F) shoots ready for acclimation out of *in vitro* culture.

Potential transgenic shoots that successfully elongated and rooted were tested by PCR for the presence of the *Hpt* transfer-DNA marker (Table 2, Figure 9). *pH35S.BBM* transformations plated on R1a media with DEX resulted in 1.7% of explants (n= 557 internodes) regenerating shoots, 90% of which tested positive for the *Hpt* marker. Interestingly, 1.2% of explants plated on DEX-supplemented media without hormones (n= 168 internodes) yielded shoots, all testing positive for the transgene (Table 2). Similar results were obtained from transformation events with the *pH35S.WUS* expression vector, though at a lower frequency. On R1a media with DEX, 0.5% of internodes (n= 740 internodes) developed shoots, with 50% testing positive for *Hpt*, while on MS-media with DEX alone 0.34% of explants (n= 575 internodes) produced shoots and 100% tested positive for the marker. As such, current transformation efficiencies (input: internodal segments; output: transgenic primary shoots) ranged from 1.2-1.6% for *pH35S.BBM* and 0.27-0.34% *pH35S.WUS.* No shoots were obtained on media without DEX in either set of transformations at the scale of explants that was used. Plants from *pH35S.BBM* and *pH35S.WUS* are currently being transitioned to *ex vitro* conditions and are awaiting additional screening for stability and expression of the transgene. To this point, we have confirmed that 15% of shoots (n= 38) from *Hpt*+

responsive explants have tested positive for the *AtBBM* gene following rooting (Figure A.1).

Table 2. Transformation efficiency of AtBBM, AtWUS and GUS transgenic lines
Following co-culture internodal explants were plated on either (1) hormone free MS-media with DEX, (2) R1a media with DEX or (3) R1a media without DEX. Responsive explants represent the number of internodes with shoots. Transformation efficiency is calculated by the number of internode explants with *Hpt* positive (*Hpt*+) shoots/total number of explants (n)*100. Values were obtained from three independent transformation events for each expression vector and media type.

	pH35S.BBM					***pH35S.WUS***					***p35S.GUS***	
Media	(n)	Responsive explants	Survival	*Hpt* +	Efficiency %	(n)	Responsive explants	Survival	*Hpt* +	Efficiency %	(n)	Responsive explants
R1	104	0	0	0	0	224	0	0	0	0	343	0
DEX	168	2	2	2	1.2	575	2	2	2	0.34		
R1+DEX	557	10	8	9	1.6	740	4	2	2	0.27		

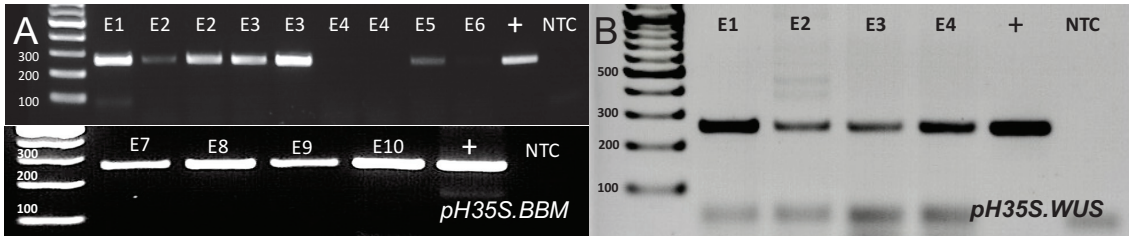

Figure 9. Early PCR screening for AtBBM and AtWUS transgenic lines.
PCR analysis of regenerated shoots using hygromycin phosphotransferase (*Hpt*) specific primers with expected product size of 251 bp. Internodal explants with shoots are numbered E1-En. Positive controls consist of *pH35S.BBM* and *pH35S.WUS* expression vectors (+) and negative controls include reactions that lack DNA template (no template control, NTC). Internodal explants E1-3 and E6-10 from *pH35S.BBM* transformations (A) and E1-4 from *pH35S.WUS* transformations (B) had shoots that tested positive for the *Hpt* transfer-DNA marker.

Improved transformation efficiency following transient expression of *AtBBM*, prompted us to evaluate the regeneration capacity of *AtBBM* positive hop plants in the absence of inhibiting *Agrobacterium* and antibiotic selection. Internodes sampled from wildtype and *AtBBM*-positive plants were selected and placed on medium R1a with DEX

and assessed for shoot regeneration following six weeks in culture. In two independent trials, we saw a 4-fold (p= 0.01) and 1.7-fold (p= 0.03) increase in shoot regeneration in *AtBBM* internodes relative to wildtype (Figure 10).

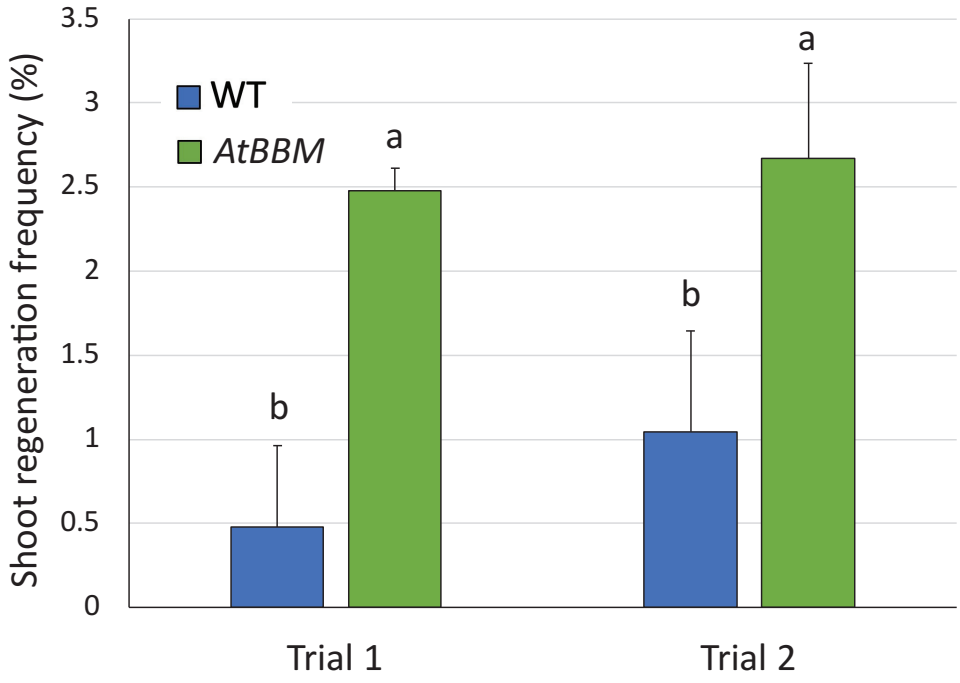

Figure 10. Regeneration response of pH35S.BBM hop plants.
Shoot regeneration frequency (number of internodes with shoots/total number of internodes*100) in *AtBBM* and wildtype (WT) Galena internodes. Average sample size n= ~50 per replicate; five replicates per trial, per genotype. Statistical significance was determined using a pair-wise Student's t-test (p<0.05). Values not represented by the same letter were found to be significantly different. Error bars = standard error.

3.1.7. Hygromycin selection

The antibiotic selection agent Hygromycin B acts by binding to the bacterial 80S ribosomal subunit, inhibiting polypeptide elongation during protein synthesis. Depending on the plant species, a large range of hygromycin concentrations can be used for selection following genetic transformation. Previous reports in hop state concentrations of 1.5-2.5 mg/L, though it is recommended to move shoots off hygromycin following shoot emergence due to unwanted toxic effects, regardless of the concentration applied (Batista et al., 2008b; Gatica-Arias & Weber, 2013; Mishra et al., 2018). We too

identified potential hygromycin toxicity in our *pH35S.BBM* and *pH35S.WUS* transformant backgrounds. When *Hpt*-positive *in vitro*-grown plants were used as a source for explants, shoots that formed from internode calli appeared pale in color, failed to elongate, and in some instances died on selection following prolonged exposure to 1.5 mg/L hygromycin. Therefore, we re-evaluated our selection method by conducting a hygromycin tolerance test in nodal cultures established from wildtype Galena and *Hpt*-positive (*AtBBM*) hop plants. We also included the genotype Nugget to identify any cultivar-specific tolerances to selection.

In three independent trials, excised nodes were plated on R2c media supplemented with increasing concentrations of hygromycin (0, 0.75, 1.5, 2.5 and 5mg/L). Cultures were assessed for shoot growth and rooting following six weeks on selection (Figure 11). All media containing hygromycin dramatically suppressed growth and inhibited shoot elongation in all three genotypes. Though significant growth depression was observed in *AtBBM* plants, despite the presence of the *Hpt* resistance gene, this genotype still out-performed wildtype cultures at all four concentrations. No difference in shoot elongation was recorded between *AtBBM* and Galena in the untreated controls, while *a* 37% (p=0.0016), 55% (p=0.0004) and 68% (p=0.053) difference in elongation occurred at hygromycin concentrations of 0.75, 1.5 and 2.5mg/L, respectively. We also identified differences in hygromycin tolerance between *Hpt* negative genotypes Nugget and Galena. Cultures differed in shoot elongation by 65% at 0.75mg/L (p=<0.0001) and 69% at 1.5mg/L (p=0.0034), while no significant differences were observed in our no treatment controls. Concentrations equal to or greater than 2.5 mg/L resulted in minimal callus growth, lack of shoot initiation and tissue death, regardless of genotype. No shoots survived 5mg/L antibiotic exposure during the six-week treatment period.

Rooting of explants occurred, but only in the *AtBBM* genotype, with 35% explants initiating roots at both 0.75 and 1.5mg/L (data not shown). Root growth was not experienced by any wildtype cultures when exposed to hygromycin, despite 100% of explants developing roots in our no-hygromycin treatment controls.

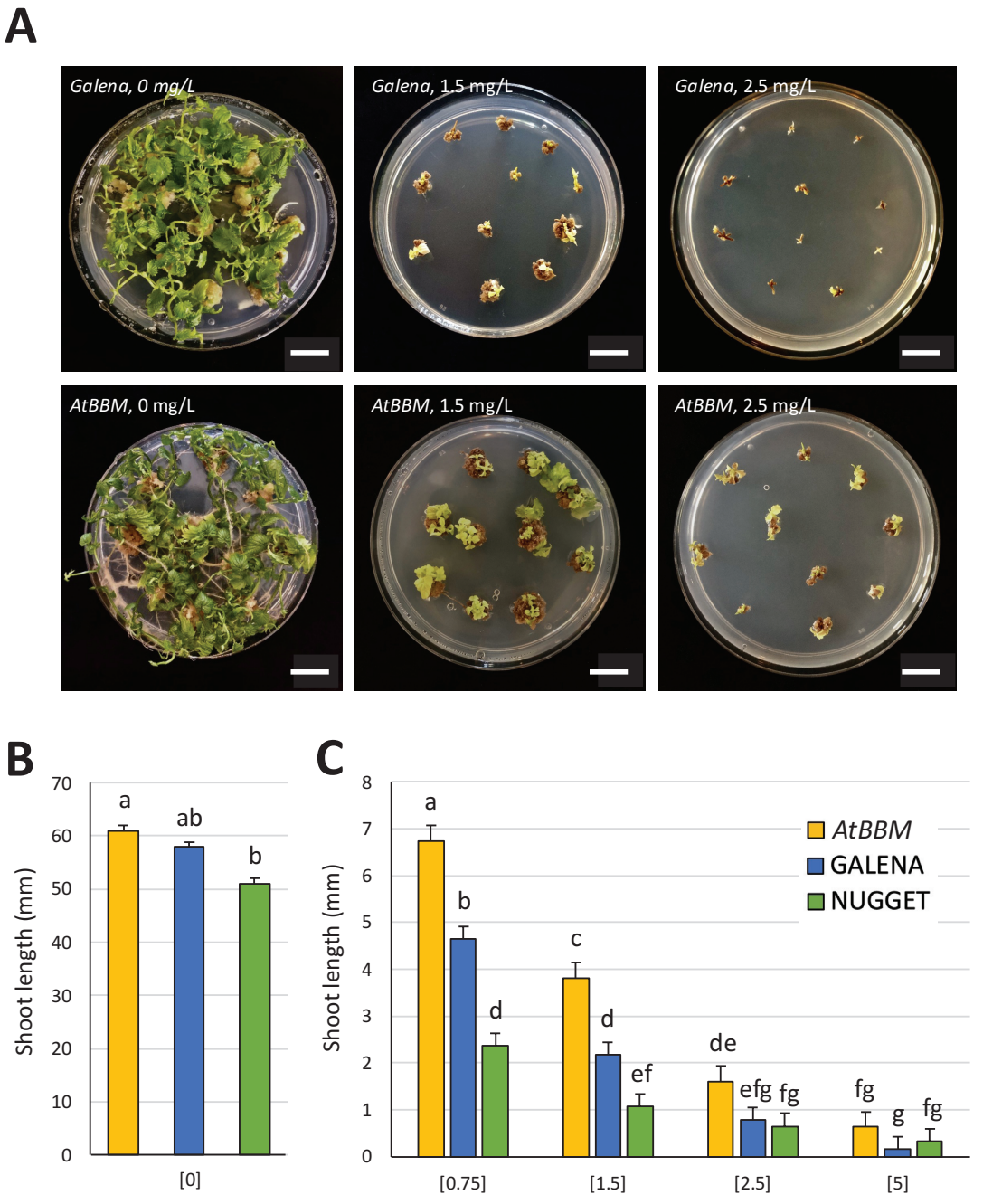

Figure 11. Hygromycin tolerance.

Shoot growth on hygromycin-free media in wildtype Galena and Nugget and *Hpt* positive *AtBBM* shoots (Galena background). Growth depression in response to hygromycin treatments (B). Inhibition of shoot elongation in genotypes Galena, Nugget and *AtBBM*, after six weeks on different concentrations of hygromycin: 0.75, 1.5, 2.5, and 5mg/L (C). Values were obtained from three independent growth trials: n=30 per genotype, per treatment, per trial. Statistical

significance was determined using a pair-wise Student's t-test (p<0.05). Values not represented by the same letter were found to be significantly different. Error bars = standard error. Scale bar = 15mm.

3.2. Gibberellin biosynthesis gene characterization

3.2.1. Growth response to exogenous GA_3 and paclobutrazol

To demonstrate that GA biosynthesis is a suitable target for gene manipulation towards semi-dwarfism in hop, we conducted a series of GA_3 and paclobutrazol (PB) watering treatments on vegetative cuttings. As the concentration of the GA biosynthesis inhibitor PB is proportional to the reduction of endogenous GA levels, it is often used to study the effect of decreasing GA concentrations on plant growth. Cuttings were rooted in rockwool, and young plantlets of similar stature were selected for treatment. The difference in shoot length was measured following 30 days of weekly GA_3 or PB supplementation (0, 2.5, or 5ppm) (Figure 12).

Shoots exposed to GA_3 experienced an increase in shoot length with increasing GA_3 concentrations. On average shoot growth increased by 36% from 0ppm-5ppm GA_3 (p=0.0278). In a separate trial, we also tested 10 and 20ppm concentrations, however, these concentrations appeared to have an inhibitory effect on growth (data not shown). PB-treated plants showed an 88% (p=<0.0001) and 97% (p=<0.0001) reduction in shoot growth at 2.5ppm and 5ppm compared to control plants. Between 2,5 and 5ppm PB treatments we found a 67% decrease in growth from an average of 18mm to 6mm, though this difference was not found to be statistically significant.

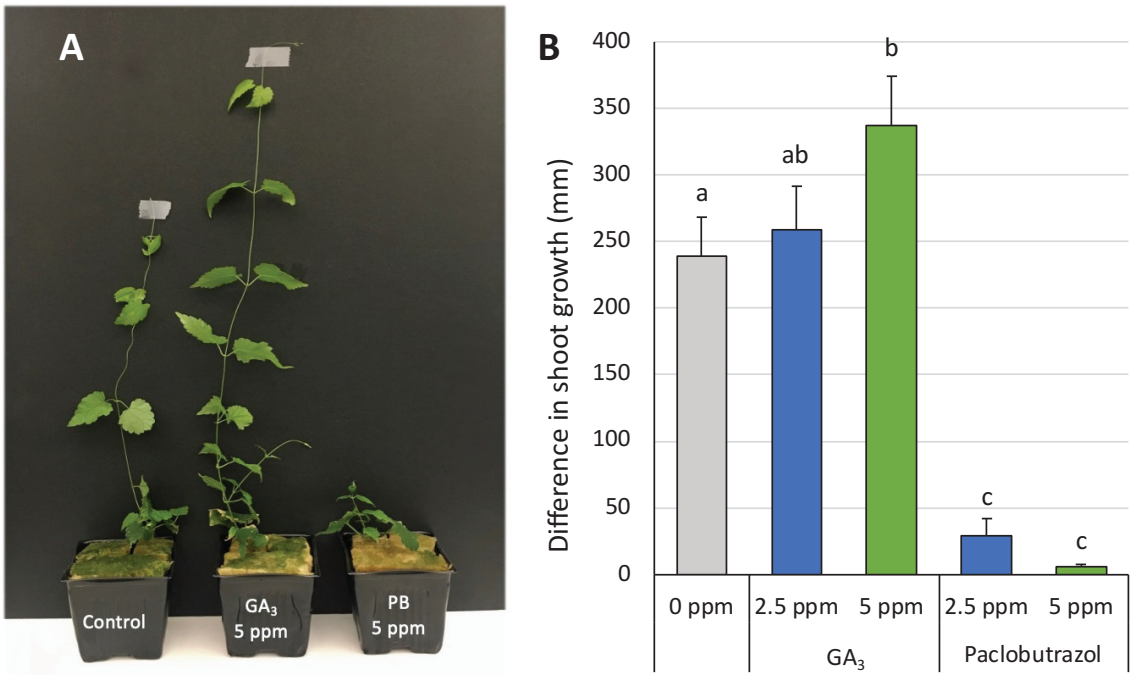

Figure 12. GA_3 and paclobutrazol (PB) applications influence hop stature.
Young hop plants following 30 days of GA_3 or PB exposure (A). Difference in hop stature following GA_3 or PB treatment: 0 (control), 2.5, or 5 ppm (B). The difference in shoot growth = final height – initial height. Measurements from two independent trials were pooled and average lengths are shown in this figure. Total sample size (n)= 18-38 plants per treatment. Independent analysis of trials provided the same results (not shown). Statistical significance was determined using a pair-wise Student's t-test (p<0.05). Values not represented by the same letter were found to be significantly different. Error bars = standard error. Abbreviations: gibberellic acid (GA_3); paclobutrazol (PB); parts per million (ppm).

*3.2.2. **Identification of GA biosynthesis genes***

To identify genes likely encoding the GA biosynthesis enzymes (*CPS*, *KS*, *KO*, *KAO*, *GA20ox*, *GA3ox* and *GA2ox*) in the hop genome, we used known cDNA and protein sequences from *Arabidopsis* and *Cannabis* to conduct a pair-wise local alignment search using the BLAST algorithms against the draft assembly for the hop genome Cascade (PRJNA562558) (Table 3).

We identified seven early GA biosynthesis gene candidates including one *CPS* (*HlCPS*), one *KS* (*HlKS*), three *KO* (*HlKO*) and two *KAO* (*HlKAO*). Protein sequences ranged from 194-527 amino acids in length. A standard protein blast in NCBI produced top sequence hits with expect values (E-values) of zero, percent query coverage of 75-99% and percent identity of 84-91%. Identified *HlCPS* and *HlKS* contained chloroplast-specific terpene synthase, N-terminal domains (IPR001906) from amino acids 67-273 (*HlCPS*) and 263-461 (*HlKS*). All three *HlKO* proteins contained the cytochrome P450 domain (IP001128) and chloroplastic ent-kaurene oxidase domain (IPR044225). Using the online tool, Plant-mPLoc, *HlCPS, HlKS,* and *HlKO* proteins were predicted to be localized to the chloroplast. As, *KAO* genes also encode cytochrome P450s, the cytochrome P450-specific domain (IP001128) was present in all our *HlKAO* protein sequences. *HlKAO* proteins subcellular localization was suggested to be the endoplasmic reticulum.

Enzymes that catalyze the later steps in the GA biosynthesis pathway, GA20ox, GA3ox and GA2ox, are members of the 2OG-Fe (II) oxygenase superfamily. Five *GA20ox* (*HlGA20ox*), three *GA3ox* (*HlGA3ox*) and five *GA2ox* (*HlGA2ox*) sequences were isolated. Protein lengths ranged from 203-392 amino acids. Output from our NCBI protein local alignment search yielded top hits with E-values of zero in all *GAox* candidates except *HlGA3ox3* (E-value=9.00E-100), though still statistically significant. Percent query coverage ranged from 85-99% and percent identity from 74-97%. All GA oxidase sequences contained the oxoglutarate/iron-dependent dioxygenase domain (IPR005123), specific to this group of enzymes. Interestingly, the highly conserved non-haem dioxygenase N-terminal domain (IPR026992) present amongst all three classes of the HlGA oxidases, was absent in *HlGA20ox2, HlGA20ox5, HlGA3ox1* and *HlGA3ox3*. *HlGAox* candidates were predicted to be localized to the cytoplasm.

Table 3. GA biosynthesis gene candidates.

Potential GA biosynthesis CDS and protein sequences were isolated using respective sequences from *Arabidopsis* and *Cannabis* as a query for BLASTp against the hop genome Cascade. Accession numbers of protein sequences referenced are from *www.hopbase.org*. Blast search results were obtained from NCBI and the top putative hits from GenBank are indicated. The expect value (E-value) describes the number of hits one can achieve by chance when searching a database of a given size. The closer the E-value is to zero, the more significant the match is. % query cover defines how much of the query sequence is covered by the target sequence and % identity describes how similar the query sequence is to the target ie. how many amino acid residues are identical.

Gene	Accession	Protein length	InterPro domains	Blast search	E-value	% Query Cover	% Identity
Early GA metabolism genes							
HlCPS	000894F.g32.t3	347	Terpene synthase	Ent-copalyl diphosphate synthase, *Trema orientale*	0	98	86.3
HlKS	001114F.g5.t1	527	Terpene synthase	Ent-kaur-16-ene synthase, *Trema orientale*	0	91	83.82
HlKO1	010748F.g4.t1	518	Cytochrome P450	Ent-kaurene oxidase, *Cannabis sativa*	0	92	90.87
HlKO2	001589F.g30.t1	370	Cytochrome P450	Ent-kaurene oxidase, chloroplastic, *Cannabis sativa*	0	99	85.09
HlKO3	001449F.g24.t2	410	Cytochrome P450	Ent-kaurene oxidase, chloroplastic, *Cannabis sativa*	0	99	89.22
HlKAO1	002003F.g6.t1	436	Cytochrome P450	Ent-kaurenoic acid oxidase 2-like, *Cannabis sativa*	0	75	86.59
HlKAO2	002125F.g12.t1	194	Cytochrome P450	Ent-kaurenoic acid oxidase 2-like, *Cannabis sativa*	0	99	86.03
GA20 oxidase enzymes							
HlGA20ox1	001736F.g16.t1	310	2OG/iron-dependent dioxygenase	Gibberellin-20 oxidase 2-like, *Cannabis sativa*	0	99	91.89
HlGA20ox2	001369F.g16.t1	203	2OG/iron-dependent dioxygenase	Gibberellin-20 oxidase 1-like, *Cannabis sativa*	0	96	96.95
HlGA20ox3	002861F.g2.t1	392	2OG/iron-dependent dioxygenase	Gibberellin-20 oxidase 1-like, *Cannabis sativa*	0	98	83.08
HlGA20ox4	001739F.g1.t1	375	2OG/iron-dependent dioxygenase	Gibberellin-20 oxidase 1-B-like, *Cannabis sativa*	0	96	94.21
HLGA20ox5	001736F.g11.t1	274	2OG/iron-dependent dioxygenase	Gibberellin-20 oxidase 2-like, *Cannabis sativa*	0	85	82.61
GA3 oxidase enzymes							
HlGA3ox1	003168F.g20.t1	205	2OG/iron-dependent dioxygenase	Gibberellin 3-beta-dioxygenase 3-like, *Cannabis sativa*	0	99	83.07
HlGA3ox2	002424F.g5.t1	368	2OG/iron-dependent dioxygenase	Gibberellin-3 oxidase, *Parasponia andersonii*	0	86	91.96
HlGAox3	000912F.g21.t1	221	2OG/iron-dependent dioxygenase	Gibberellin 3-beta-dioxygenase 1-like, *Cannabis sativa*	9.00E-100	96	74.35
GA2 oxidase enzymes							
HlGA2ox1	000411F.g28.t1	351	2OG/iron-dependent dioxygenase	Gibberellin 2-beta-dioxygenase 2-like, *Cannabis sativa*	0	96	86.04
HlGA2ox2	000063F.g71.t1	364	2OG/iron-dependent dioxygenase	Gibberellin-2 oxidase, *Trema orientale*	0	98	79.11
HlGA2ox3	005880F.g9.t1	336	2OG/iron-dependent dioxygenase	Gibberellin 2-beta-dioxygenase, *Cannabis sativa*	0	99	88.36
HlGA2ox4	000402F.g20.t1	382	2OG/iron-dependent dioxygenase	Gibberellin 2-beta-dioxygenase 8-like, *Cannabis sativa*	0	90	89.58
HlGA2ox5	005364F.g4.t1	328	2OG/iron-dependent dioxygenase	Gibberellin 2-beta-dioxygenase 1-like, *Cannabis sativa*	0	99	95.77

Phylogenetic trees were constructed to evaluate homology and evolutionary distance between protein sequences predicted in hop and respective sequences in *Arabidopsis* and *Cannabis*. The early GA biosynthesis enzymes showed two distinct branches separating terpene synthases and cytochrome P450s (Figure 13.A). Terpene synthases were further divided into *CPS* and *KS*, while Cytochrome P450s branched into *KO* and *KAO*. Ten conserved motifs were identified using the online software MEME (Figure 13.A, Figure A.2). Each enzyme classification was found to have distinct motif compositions. Motifs 1 and 4 were shared amongst all cytochrome P450s, while motifs

2, 3, and 6 were specific to KO and motifs 5 and 10 were only found in KAO. A single conserved motif was identified amongst all terpene synthases (motif 8), while motif 7 was present in the *KS* sequences alone. However, motif 7 did not appear to be evolutionarily conserved within KS enzymes as it was also possessed by KAO. Motif 8 was found across all four early GA biosynthesis enzymes.

Phylogenetic analyses of the GAox enzymes identified in hop, *Arabidopsis* and *Cannabis*, showed a clear pattern of organization based on function. Three groups were identified separating GA20ox (Group I), GA3ox (Group 2) and GA2ox (Group 3) proteins (Figure 13.B). A subset of GA2ox sequences obtained from *Arabidopsis* and *Cannabis* showed relatively low sequence similarity with other GA2ox proteins, forming a fourth group composed of *AtGA2ox7,8* and *CsGA2ox4,5,6*. Various reports show that GA2ox proteins fall into two classes of enzymes based on their substrate, C19-GA, or C20-GA. *AtGA2ox7* and *AtGA2ox8* belong to the C20 class of GA2ox enzymes. As such, homologs identified in the same clade may also fall into this functional classification. Interestingly, no hop GA2ox proteins were found in the C20 subgroup.

Both common and distinguishable motif compositions were distributed across the GAox proteins (Figure 13.B, Figure A.3). Motifs 1-4,6 and 12 were possessed by all four classes and may be a common feature of the 2OG-Fe (II) oxygenase family. Motifs 7, 8 and 10 were conserved across C19-GA2ox, while motif 15 was specific to C20-GA2ox. The signature motifs for GA20ox appeared to be motifs 13 and 14. Both GA20ox and GA3ox groups possessed motif 5, though absent in *HlGA20ox2*,*5* and *HlGA3ox1,3*. These hop enzymes were also missing motif 6, despite being a common signature amongst all GAox proteins analyzed. As such, isolated *HlGA20ox2*,*5* and *HlGA3ox1,3* sequences from the representative hop genome may be incomplete. Their sequence alignments appeared truncated in comparison to their respective homologs in *Arabidopsis* and *Cannabis*

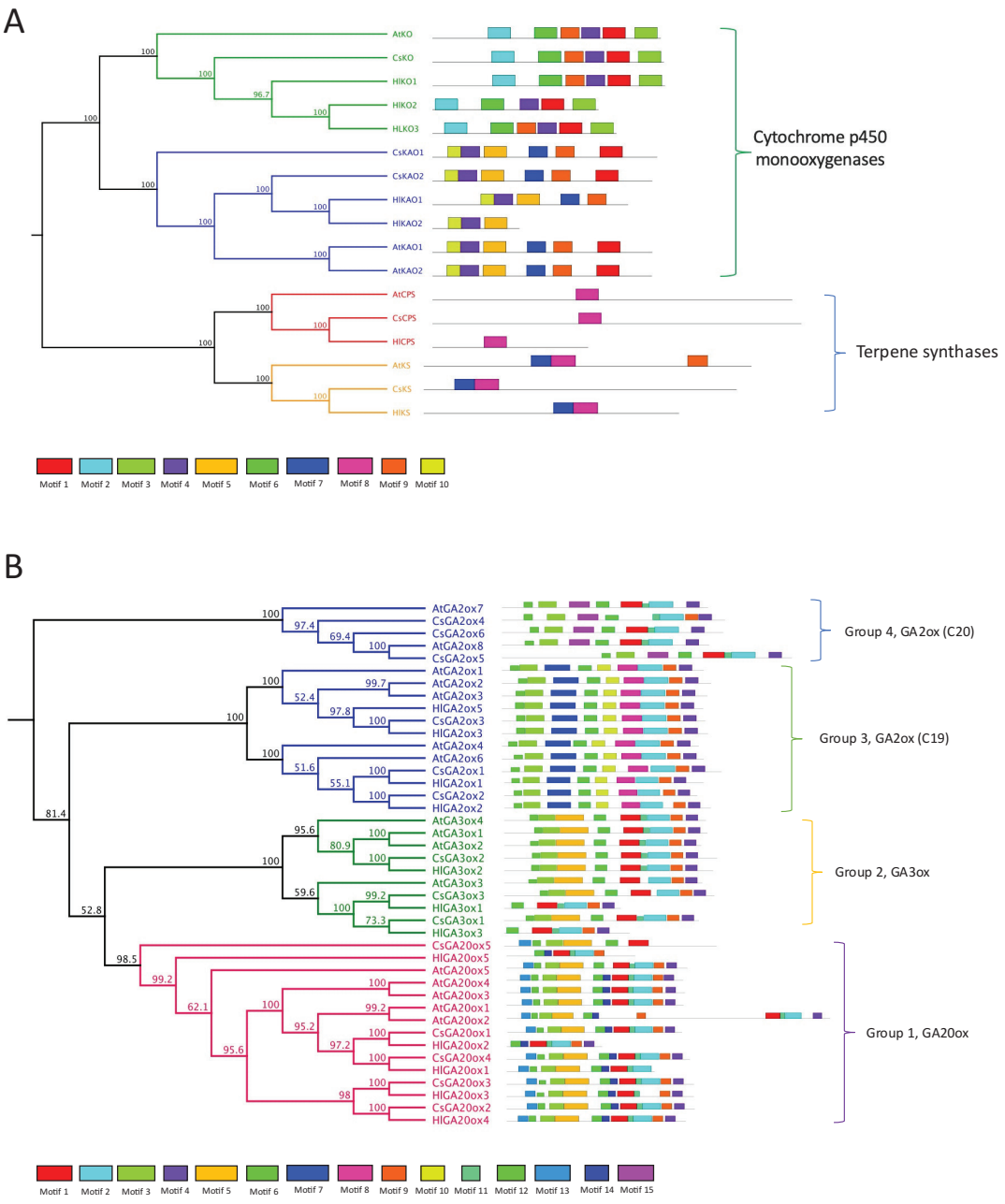

Figure 13. Phylogenetic relationships and conserved protein motifs.

Phylogenetic relationships and conserved protein motifs amongst (A) early GA biosynthesis genes and (B) GA oxidase genes identified in hop. Protein Sequences were aligned with Muscle and phylogenetic trees were constructed in Geneious using the UPGMA algorithm. Bootstrap values were obtained using 1000 replicates. Conserved protein motifs were identified using the online software MEME and are represented by different colored boxes. The length and position of each box corresponds to the length of each motif and its position along the protein sequence. Abbreviations: Hl, *Humulus lupulus*; Cs, *Cannabis sativa*; At, *Arabidopsis thaliana*.

3.2.3. ***Tissue-specific expression of GA oxidase candidates***

Following identification of putative GA biosynthesis genes, we measured spatial expression of the *HlGA20ox* and *HlGA3ox* candidates using quantitative real-time polymerase chain reaction (qRT-PCR) on cDNA synthesized from roots, internodes, leaves, and female cones. Recall that these gene candidates were chosen based on the semi-dwarf phenotype produced in *Arabidopsis* when single genes are knocked out. Comparative expression was assessed for each gene individually as well as across each gene family via the 2^-(ΔΔCt) method. The lowest expression value for each analysis functioned as the control.

HlGA20ox candidates were broadly expressed, but also showed distinct tissue specificity. *HlGA20ox1* was predominantly expressed in apical internodes, immature leaves, and young roots (Figure 14.A), while *HlGA20ox2* was significantly expressed in apical internodes alone (p=0.0007) (Figure 14.B). *HlGA20x3* had relatively low expression levels across tissue types and failed to be detected in basal internodes and mature leaves (Figure 14.C). The lack of expression was confirmed through gel electrophoresis, which yielded absent bands. The *HlGA20ox4* homolog was distinctly expressed in female cones, with an over 200-fold increase in expression relative to the designated control (p=<0.0001) (Figure 14.D). Lastly, *HlGA20ox5* showed a similar expression pattern as *HlGA20ox1*, though expressed at relatively lower levels (Figure 14.E,F). When comparing gene expression across the hop *GA20ox* candidates, a general trend was high expression in young tissues relative to those that had slowed in development (Figure 14.F). Overall, gene expression was detected at low levels in basal internodes and mature leaves, while apical internodes, immature leaves were favored. All *HlGA20ox* genes except for *HlGA20ox4*, exhibited weak expression in female cones.

Among the three *HlGA3ox* candidates, *HlGA3ox1* and *HlGA3ox3* exhibited broad expression, but at relatively low levels (Figure 15.A,B). *HlGA3ox2* appeared to be the dominant *GA3ox* protein, exhibiting high expression across all tissue types, yet favoring roots and female cones (Figure 15.C). Relative to the set control, an approximate 60-fold (p=<0.0001) and 25-fold (p=0.014) increase in gene expression was measured in roots and cones, respectively. Consistent with *HlGA20ox* candidates, the *HlGA3ox* genes showed weaker expression in matured tissue types compared to those still experiencing growth (Figure 15.D).

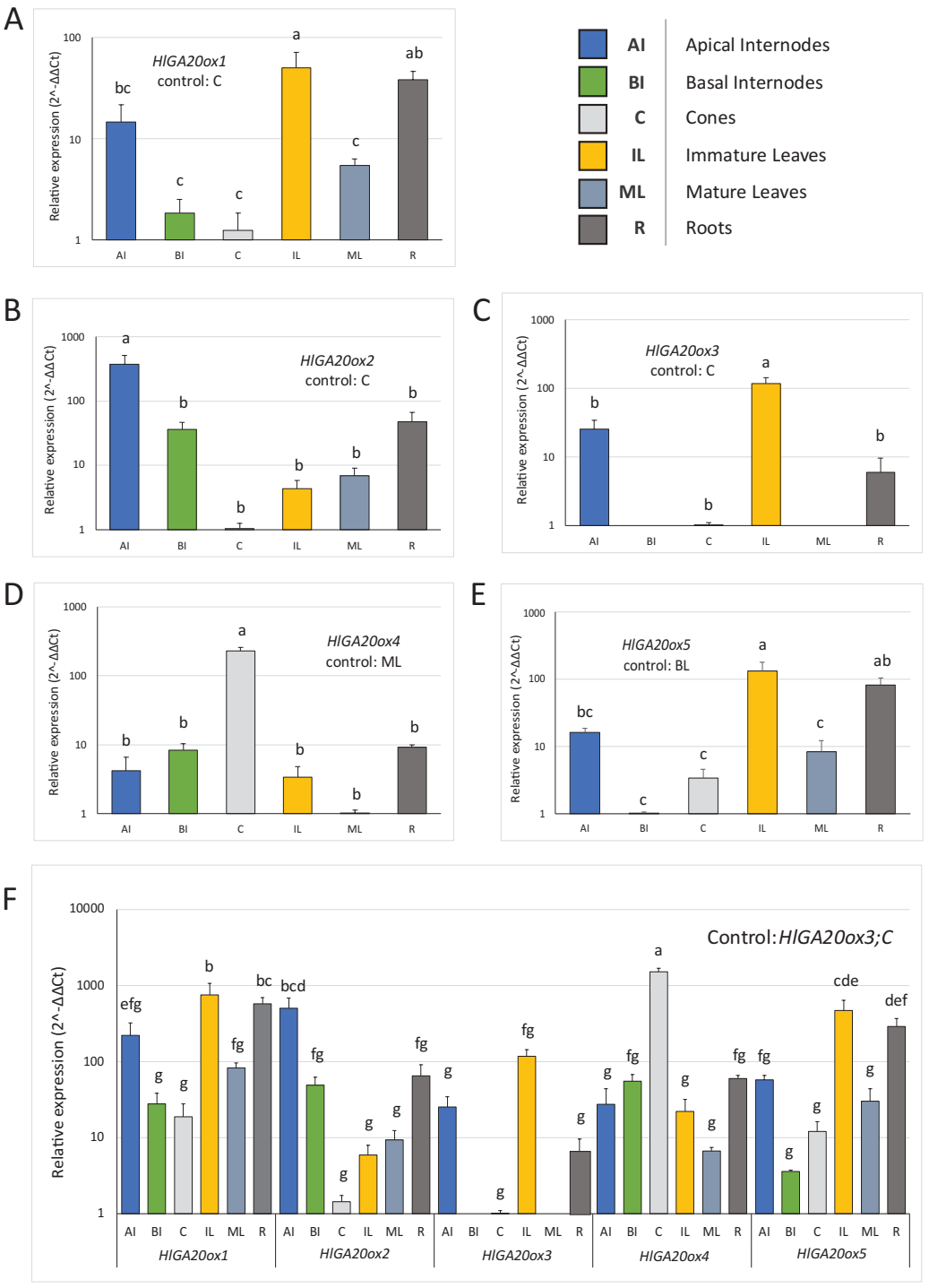

Figure 14. Differential gene expression of HlGA20ox candidates.

qRT-PCR was performed on cDNA synthesized from apical internodes (AI), basal internodes (BI), female cones (C), immature leaves (IL), mature leaves (ML) and young roots (R). Tissue-specific expression was assessed for each individual gene (A-E) as well as across the *HlGA20ox* gene

family (F). Expression levels of *HlGA20ox1-HlGA20ox5* were quantified via the 2^-(ΔΔCt) method. The Ct values of each gene were an average of three biological replicates and three technical replicates. Statistical significance was determined using a pair-wise Student's t-test (p<0.05). Values not represented by the same letter were found to be significantly different. Error bars = standard error; Y axis = log10 scale.

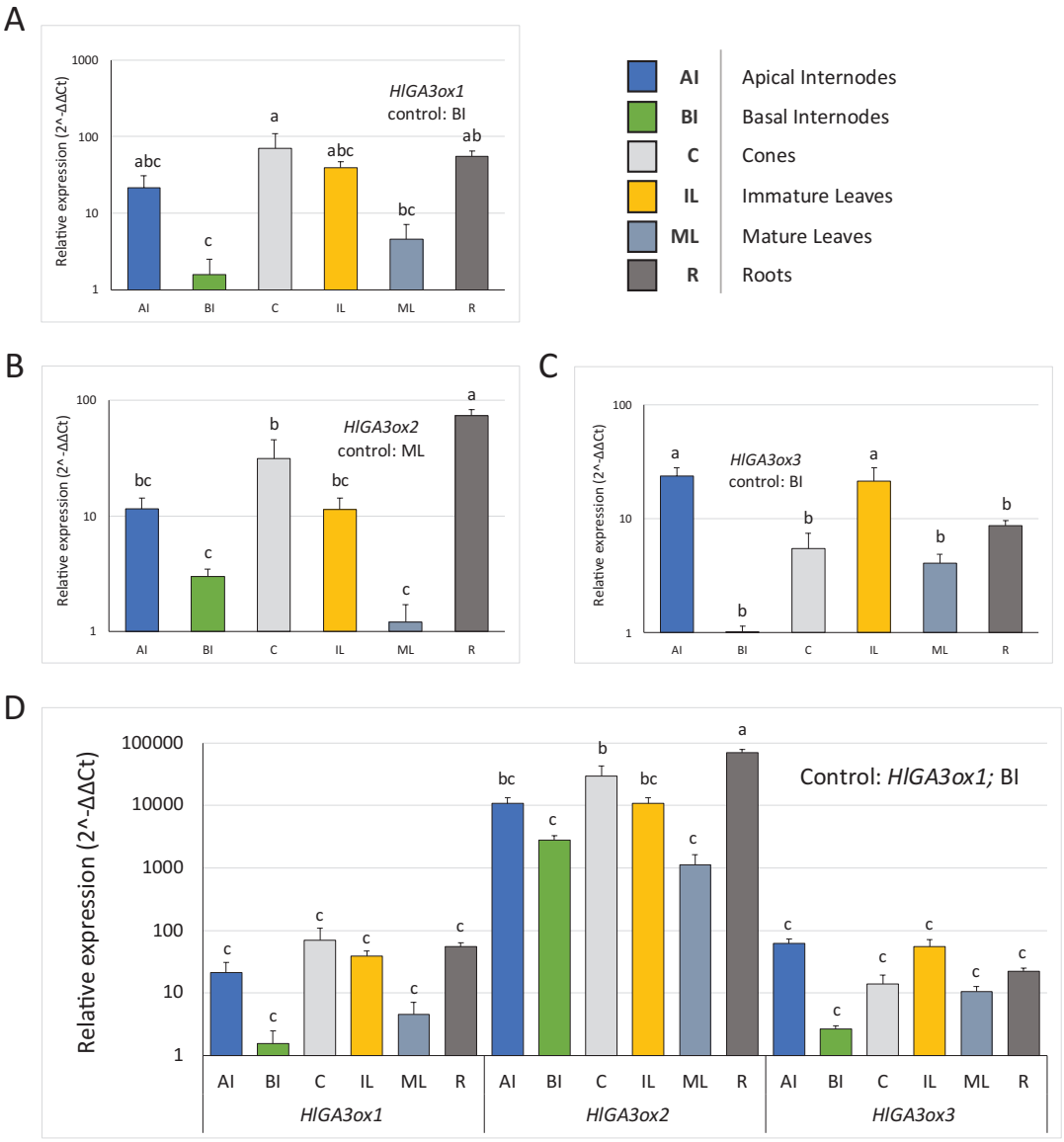

Figure 15. Differential gene expression of HlGA3ox candidates.
qRT-PCR was performed on cDNA synthesized from apical internodes (Al), basal internodes (Bl), female cones (C), immature leaves (IL), mature leaves (ML) and young roots (R). Tissue-specific expression was assessed for each individual gene (A-C) as well as across the *HlGA3ox* gene family (D). Expression levels of *HlGA3ox1-HlGA3ox3* were quantified via the 2^-(ΔΔCt) method.

The Ct values of each gene were an average of three biological replicates and three technical replicates. Statistical significance was determined using a pair-wise Student's t-test ($p<0.05$). Values not represented by the same letter were found to be significantly different. Error bars = standard error; Y axis = log10 scale.

3.2.4. Transformation with T-DNA for CRISPR-induced knockouts of HlGA20ox1 and HlGA20ox2

To identify which *GAox* homologs in the hop genome would be ideal targets towards semi-dwarfism, we prioritized *HlGAox* candidates based on high expression in internodes and low expression in female cones. Both, *HlGA20ox1* and *HlGA20ox2* fit these criteria (Figure 14.A,B). Moreover, each candidate showed the highest sequence similarity with *AtGA20ox1* and *AtGA20ox2* (Figure 13.B), which function redundantly to promote internode elongation. *HlGA20ox1* exhibited an 81% and 78% positive identity score when aligned to *AtGA20ox1* and *AtGA20ox2*, respectively. Likewise, *HlGA20ox2* showed an 83% and 85% positive identity score following the same alignments.

Two gRNAs were designed per *HlGA20ox* target gene using the online software CRISPR RGEN and the Shinsuwase reference genome (Table 4). Both gRNAs designed for *HlGA20ox1* target the highly conserved non-haem dioxygenase N-terminal domain (IPR026992), while gRNA1 specific to *HlGA20ox2* targets the oxoglutarate/iron-dependent dioxygenase domain (IPR005123) and gRNA2 targets just upstream of that. Target sites were located on the positive strand of DNA, had a GC content between 52-63% and generated out-of-frame scores that ranged from 62-83. As a general rule, out-of-frame scores above 66 are recommended to avoid unwanted in-frame deletions (CRISPR RGEN). Furthermore, both Shinsuwase and Cascade genomes were screened for potential off-target effects. No off-target sites were identified in either genome.

Table 4. gRNA target identification.

Target sequences were identified using the online software CRISPR RGEN for *GA20ox* candidates *HlGA20ox1* and *HlGA20ox2*. gRNA spacers are noted in black and PAM sequences (NGG) are highlighted in red. Target sequences are read 5' to 3' and were located on the positive strand of DNA. The out-of-frame score = the ratio of out-of-frame pattern scores over the sum of all the patterns scores.

Gene	gRNA	Target sequence (5'→3')	Strand	GC %	Out of frame score
HlGA20ox1	1	GGAAAGTAGGGGAACACTG**CGG**	+	52.6	83.4
	2	GGCTTCTTGTCCGGTGACC**CGG**	+	63.2	72.5
HlGA20ox2	1	CAGATCTAACACTGGGGAC**TGG**	+	52.6	73.3
	2	AGGATCTCACTTGGGGTGA**TGG**	+	52.6	62.4

Several intervarietal single nucleotide polymorphisms (snps) have been identified in hop. As such, genomic DNA flanking our *HlGA20ox* target sequences were PCR-amplified and sequenced from our preferred genotype, Galena. In each case, target sequences were identical to those obtained from CRISPR RGEN (Figure 16.A). As the hop reference genome available for gRNA design was unannotated, we confirmed target sites occurred within the exons of each gene by cross-referencing sequence data obtained from Galena gDNA and cDNA with the Cascade genome on HopBase (Figure 16.B). Spacer sequences were successfully introduced into the gRNA cloning site of the *pHSE401* plasmid, which was confirmed through colony PCR and Sanger sequencing (Figure 16.C).

Purified plasmids, *pHSEGO1.1*, *pHSEGO1.2*, *pHSEGO2.1* and *pHSEGO2.2*, were independently electroporated into the *Agrobacterium* strain EHA105. The first attempt at transformation was carried out using vectors *pHSEGO1.1* and *pHSEGO2.1*. Constructed vectors were transformed to Galena-derived internodes (*pHSEGO1.1,* n= 688 and *pHSEGO2.1*, n= 483) using *Agrobacterium*-mediated transformation (see methods). However, we were only able to generate transgenic callus with the specified sample size (Figure B.3). To improve transformation efficiency we took two approaches: (1) alleviate hygromycin toxicity by reducing hygromycin selection from 1.5 mg/L to 0.75 mg/L and (2) introduce the *35S::AtBBM:GR* expression cassette into our *pHSE* vectors as a means of positive selection. The *35S::AtBBM:GR* insert was PCR-lifted out of *pH35S.BBM* and cloned into *pHSEGO1.1* and *pHSEGO2.1* via sequence and ligase

independent cloning (SLIC) (Figure 17). Corresponding *pHSE* vectors that include *AtBBM* will be referred to as *pGO1BBM* and *pGO2BBM*, respectively.

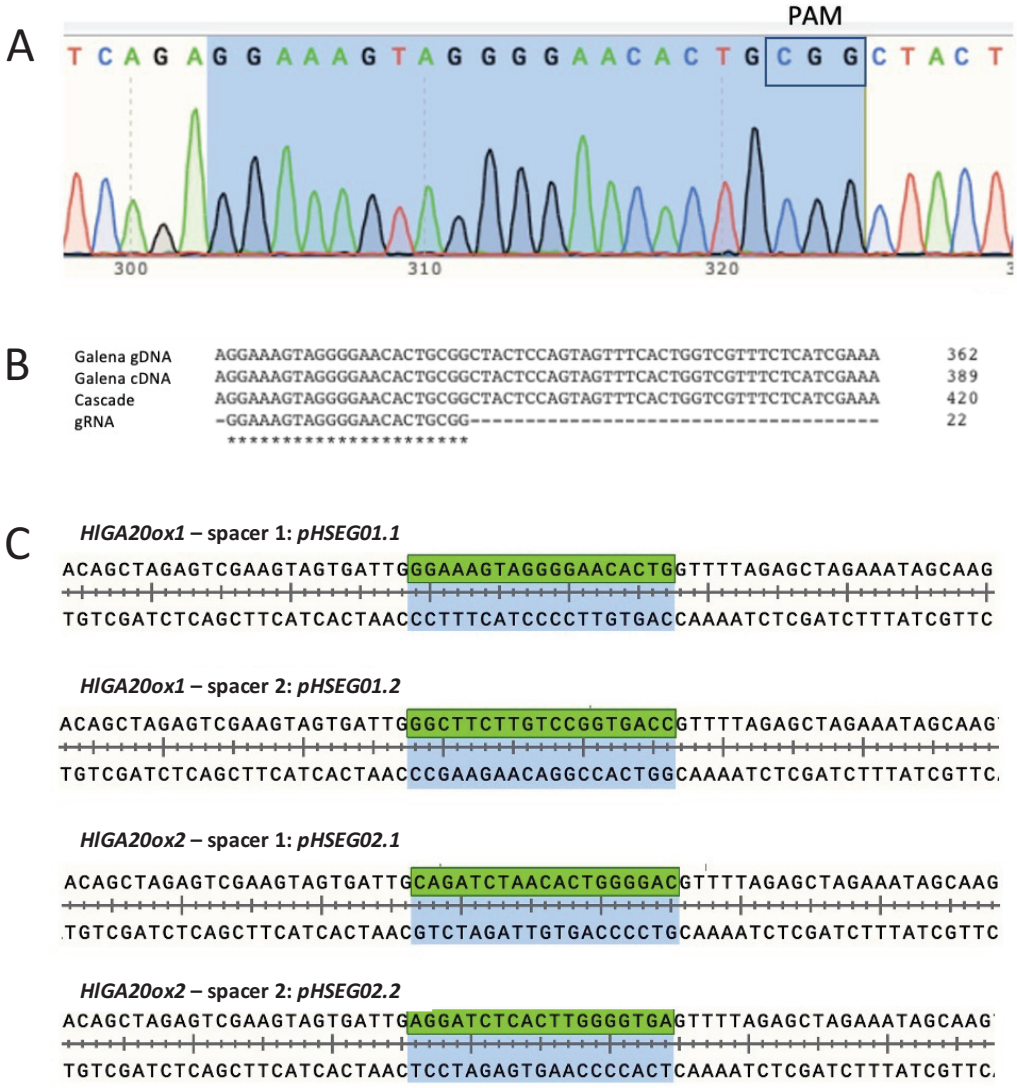

Figure 16. gRNA design and pHSE401 cloning.

Chromatogram obtained from the working cultivar Galena showing absence of SNPS at *HlGA20ox1,* gRNA target site 1 (A). Representative sequence data obtained from both gDNA and cDNA cross-referenced to the Cascade genome available on HopBase, confirming the target site occurs within the exon of a gene (B). Confirmation of gRNA spacer sequence cloning in pHSE401 (C).

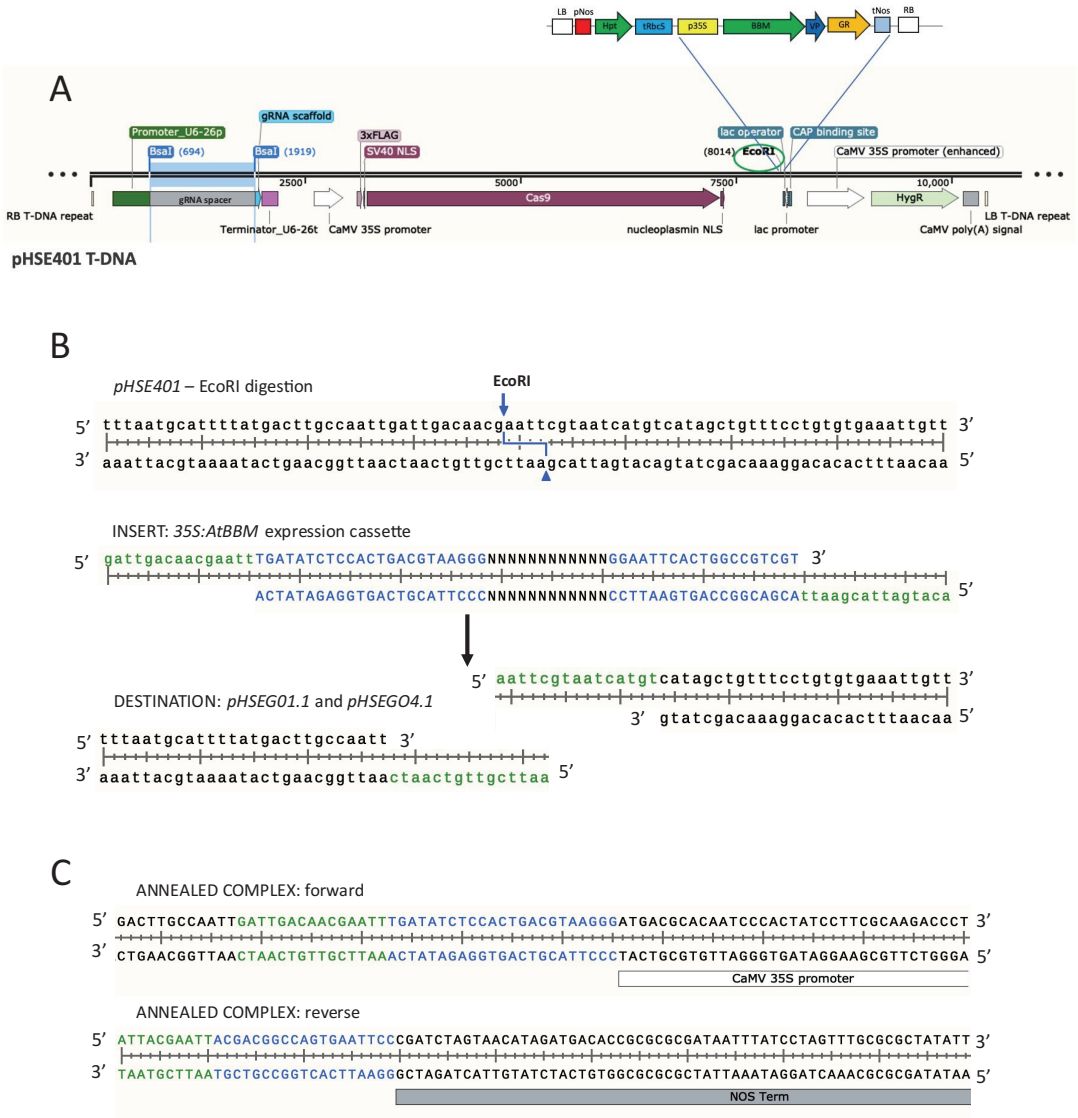

Figure 17. SLIC cloning of 35S::AtBBM:GR into pHSEG01.1 and pHSEGO2.1.
Map of *pHSE* T-DNA and insert with cloning site indicated (A). Schematic overview of SLIC cloning. *pHSE* was digested with EcoRI. The insert containing *35S::AtBBM:GR* (3292 bp; p35S to tNos) was lifted out of *pH35SBBM* using primers (blue) that contain 15 bp extensions (green) homologous to the EcoRI-cleaved *pHSE* vector. Complementary 5'-overhangs were generated by T4 DNA polymerase treatment (B). The annealed complex was confirmed via Sanger sequencing using primers SLICseq (forward) and M13/pUC (reverse) (C). Representative sequence data shown was obtained from *pHSEGO1.1*, now referred to as *pGO1BBM*. All figures were generated with the help of SnapGene.

To provide additional evidence that ectopic expression of *AtBBM* in hop can be used as a tool to improve transformation efficiency, side-by-side transformations using *pHSE* vectors with and without *35S::AtBBM:GR* were completed (Table 5). All transformations involving *35S::AtBBM:GR* were plated on shoot regeneration media (R1a) supplemented with 5μM DEX in order to activate the *AtBBM* protein. Shoots appeared to regenerate both directly and indirectly when transformed with *AtBBM*-containing vectors, while only indirect regeneration was observed when *AtBBM* was absent. When targeting *HlGA20ox1* for deletion, 0.35% of explants regenerated shoots with *pGO1BBM* (3 trials, n= 1130), while only 0.12% of explants were responsive with *pHSEGO1*. For the most part, shoots targeted with either vector lacked the ability to complete development. Shortly after regeneration, shoot primordia halted growth, turned brown and died. Survival aside, regeneration frequencies observed in transformations targeting *HlGA20ox2* yielded similar results; 0.42% of explants expressed shoots with *pGO2BBM* (3 trials, n= 959) and 0.11% of explants regenerated shoots with *pHSEG02.1* (3 trials, n= 866).

As different gRNAs have different efficiencies, we also targeted *HlGA20ox1* and *HlGA20ox2* with *pHSEGO1.2* and *pHSEGO2.2*, respectively (table 4). Transformation with *pHSEGO1.2* resulted in 1% of explants expressing shoots (1 trial, n= 299), while no shoots were obtained with *pHSEGO2.2* at the given scale (1 trial, n= 318).

To date, several potential transgenic lines have been collected from each transformation (Figure 18.A). Transformations without the help of *AtBBM* produced an average of 4.5 shoots per explant (n= 5). Conversely, experiments that utilized *pHSE* with *AtBBM* had explants overwhelmed with independent shoot primordium (too many to count). As shoot regeneration can be a lengthy process in hop (4-6 months), we are currently waiting for shoots to reach a state where they can be properly genotyped and screened for *GA20ox* deletions. To this point, we have conducted early screening for T-DNA markers, *Hpt* and *Cas9*, on shoots obtained from a single internode transformed with *pGO2BBM* (Figure 18.B). Out of 32 plants, 28% of shoots tested positive for the transgene; however, none of which to date contained mutations in the *HlGA20ox2* target.

Table 5. Summary of transformations targeting HlGA20ox candidates.

Efficiency (%) is calculated by the number of internodes with shoots/total number of internodes (n)*100. Values from side-by-side transformations with *pHSE* vectors with *35S::AtBBM:GR* (*pGO1BBM* and *pGO2BBM*) and without *35S::AtBBM:GR* (*pHSEGO1.1* and *pHSEGO2.1*) were obtained from three transformation trials. Values obtained from expression vectors *pHSEGO1.2* and *pHSEGO2.2*, which contain alternative gRNAs, were collected from a single trial.

		HlGA20ox1					*HlGA20ox2*		
Plasmid	n	Responsive explants	Survival	Efficiency %	Plasmid	n	Responsive explants	Survival	Efficiency%
pHSEG01.1	855	1	0	0.12	*pHSEGO2.1*	866	1	1	0.11
pGO1BBM	1130	4	1	0.35	*pGO2BBM*	959	4	4	0.42
pHSEG01.2	299	3	3	1.0	*pHSEGO2.2*	318	0	0	0

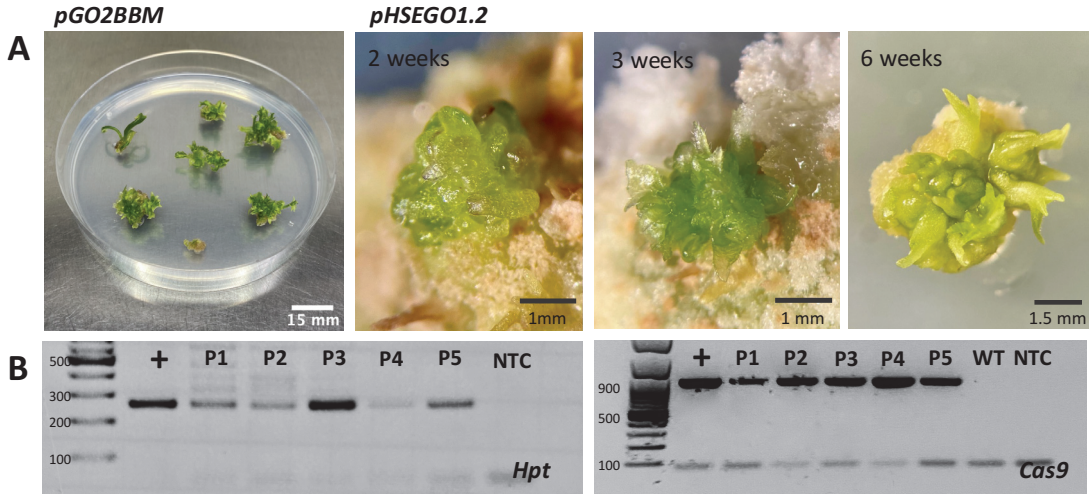

Figure 18. Screening of potential shoots for pHSE T-DNA markers.

Collected shoots from *pG04BBM* and *pHSEGO1.2* transformations currently awaiting genotyping and screening for *GA20ox* deletions (A). PCR-confirmation of transgene in shoots obtained from *pGO4BBM* transformations using both *Hpt* and *Cas9*-specific primers. Expected product: *Hpt* = 251-bp; *Cas9* = 904-bp.

Chapter 4. Discussion

4.1. Shoot regeneration response

4.1.1. Hormone use, explant type and genotype influence hop organogenesis

An efficient regeneration system is a pre-requisite for attaining transgenic plants. Few papers have been published dealing with *de novo* shoot regeneration in hop; largely because of experienced recalcitrance and genotype-specific requirements. To date, *de novo* shoot regeneration has been carried out in only a limited number of genotypes: Challenger (Heale et al., 1989), Brewers Gold (Batista et al., 1996; Gurriarán et al., 1999), Nugget (Gurriarán et al., 1999), Tettnanger (Horlemann et al., 2003), Eroica (Batista et al., 2008a) and Osvald-72 (Matoušek et al., 2012; Mishra et al., 2018; Smýkalová et al., 2001). Here we demonstrated the first example of successful shoot and root regeneration in the cultivar Galena. Consistent with previous reports, explant source, media composition and hop genotype greatly influenced organogenesis.

All explants from both Nugget and Galena were capable of callus induction on all four shoot regeneration media assayed. Though, a consistent distinction between genotypes involved the evident browning of callus derived from Nugget. Tissue browning, which occurs from the accumulation of phenolic compounds, is a significant problem in culture as it inhibits plant growth and reduces cell proliferation (Chugh et al., 2009; Ling et al., 2007). Phenolic compounds are secreted during the isolation of explants and the oxidation of these compounds on surface wounds results in necrosis, inhibiting nutrient uptake from the culture media (Irshad et al., 2017). The presence and absence of callus browning in the two cultivars is a potential consequence of phenolic secretions specific to each genotype. Furthermore, this could in part, explain the various degrees of recalcitrance observed between the two cultivars. In alternative plants species, browning effects have been avoided by including adsorbing compounds (charcoal) and anti-oxidants (citric acid and ascorbic acid) to improve *in vitro* performance (Irshad et al., 2017; Rani & Dantu, 2016; Roh & Kim, 2014). Future work should evaluate the extent phenolic secretion influences hop organogenesis and determine whether these anti-browning agents can be used to improve regeneration in Nugget.

When comparing regeneration potential between genotypes, Galena internodes on TDZ and IAA media (R1a) presented the highest capacity for shoot regeneration (Table 1), hence we choose this combination for future transformation assays. This hormone combination was also shown to be ideal for the direct regeneration of the cultivar Tettnanger (Horlemann et al., 2003). Although we were able to induce shoots in Nugget, it occurred at relatively low frequencies. Despite this, the cultivar appeared to be more responsive to different culture conditions. Nugget developed shoots on three of the four media tested (Galena responded to two) and all three explant types responded with shoot formation. Though callus quality of Nugget may influence regenerative potential, a more dominant factor explaining the variation in genotype response is likely a result of intraspecific differences in endogenous hormone biosynthesis and perception. Recall that a determining factor between shoot and root regeneration is the correct ratio of cytokinin to auxin (Skoog & Miller, 1957). Hop appears to deviate from the norm in the comparison between medium with 4.6μM zeatin and 1.4μM IAA (R1b) and with 4.6μM zeatin alone (R1c) (Table 1). We chose to test R1b in our two cultivars based on its efficiency in facilitating shoot regeneration in the hop Osvald-72 (Mishra et al., 2018). However, this combination resulted in root rather than shoot formation in both Nugget and Galena. Once IAA was removed from medium R1b, we were able to get shoot induction in internodes and petioles in both cultivars, while root formation was nearly abolished. Thus, despite the high cytokinin to auxin ratio in R1b, roots rather than shoots formed. In addition, cytokinin alone resulted in shoot formation. Taken together, these results suggest either a high endogenous concentration of auxins or a high sensitivity to auxins in the explants of these varieties, easily resulting in root fate of formed meristems. This in turn could explain, at least in part, the observed recalcitrance of hop to cytokinin-induced *de novo* shoot formation. If so, inhibition of auxin biosynthesis or perception could possibly result in higher frequency of de novo shoot formation. It should be mentioned that regeneration of plants via first root then shoot formation is typically not an option due to poor shoot formation from roots in most species.

The influence of explant source on *in vitro* culture is well recognized (Bhatia et al., 2005; Christopher & Rajam, 1996; Khaliluev et al., 2014). Here we noted differences in shoot regeneration between *in vitro* internodes, petioles, and leaves, which could be a consequence of varying degrees in organogenic competence of the cells that make up these source tissues. Ideal explants for regeneration include embryonic tissues such as

the cotyledon or hypocotyl, but these are typically avoided in hop culture because of the lack of seed availability and difficulties with seed germination and sterilization. The most commonly utilized explant for shoot regeneration in hop is *in vitro* internodes (Horlemann et al., 2003; Mishra et al., 2018; Okada et al., 2003; Schwekendiek et al., 2005). Though regeneration capacity of alternative tissues have been evaluated and deemed less efficient, comparisons were made using a single regeneration medium (Horlemann et al., 2003) and the hormone usage may not have been ideal for each given explant type. Based on our screening of internode, petiole and leaf tissue, an explants capacity for regeneration may rely, in part, on the hormones used in culture (Table 1). For example, Galena internodes showed the highest capacity for shoot regeneration in response to TDZ and IAA (R1a), yet Galena petioles were more suitable with zeatin exposure. Furthermore, the only tissue capable of shoot induction on BAP-containing media (R1d) were petioles derived from Nugget, indicating that each explant type (in addition to genotype) requires a different hormone balance.

In any regeneration system, the quality of shoots is just as important as the quantity. Though our chosen regeneration media (R1a) was efficient for shoot induction, developing shoots appeared malformed and vitrified (Figure 2). Vitrification mechanisms are unclear, but it can be caused by exogenous hormone supplementation and incorrect cytokinin to auxin ratio (Ziv, 1991). In our case, we assumed that observed shoot malformities were a consequence of the TDZ in our media. Though TDZ has been successfully used to overcome regeneration recalcitrance in a wide range of plant species, several drawbacks have been associated with its use in culture, including the development of abnormal leaf morphology, fasciated shoots, and swollen shoot bases (Reviewed in Dewir et al., 2018). These TDZ-induced effects were evident when comparing TDZ with cytokinins zeatin and BAP in our nodal cultures (Figure 3). Hence, when developing our workflow from regeneration to *ex vitro* acclimatization, we subsequently subcultured TDZ-induced shoots to an alternative medium containing BAP (R2c). This additional step resulted in the development of shoots with normal morphology and healthy tissue. Furthermore, culture on R2c increased axillary shoot proliferation and induced shoot elongation and rooting in hop culture (Figure 3).

4.1.2. Pluronic F68 enhances shoot regeneration potential

Similar to previous accounts, hop regeneration in Galena and Nugget was difficult to achieve. To date, we are unaware of any regeneration studies combining the use of plant growth regulators and chemical regeneration enhancement in hop. Our results show that the non-ionic surfactant P-F68 enhances hormone-based shoot regeneration 2.4-3 fold from hop internodes at concentrations 0.01-0.1% in the cultivar Galena (Figure 6.A). The regeneration frequency of control plants was low in these trials, probably because of long time in *in vitro* cultivation, so it is possible that the enhancement provided by P-F68 is different in more responsive material. Regenerated plants appeared morphologically normal in appearance when compared to controls, suggesting cultures exhibited good tolerance during exposure. P-F68 significantly enhanced shoot regeneration in both jute and citrus within a comparable concentration range (Cancino et al., 2001; Khatun et al., 1993). Like hop, both species are known for their high recalcitrance to *in vitro* organogenesis. Interestingly, the study conducted in jute noted that when P-F68 was present, browning effects and necrosis of explants was reduced and frequent sub-culturing was not required; a consequence with high practical value (Khatun et al., 1993). We too noticed less browning of P-F68-exposed hop cultures compared to controls (Figure 6.B). Although the activity of P-F68 is unclear in plant cell culture, some evidence suggests the surfactant inhibits the accumulation of polyphenolic compounds by altering the activity of key metabolic enzymes (Boboc Oros et al., 2022; Kumar et al., 1991). In our present study we focused our efforts on the regeneration of the cultivar Galena as this genotype was chosen for transformations. However, as Nugget experienced more severe browning effects in culture, future analyses will determine if P-F68 improves culture quality and shoot regeneration in this alternative hop genotype.

4.1.3. Morphogenic regulators BABYBOOM and WUSCHEL improves genetic transformation

Much progress has been made in understanding the molecular basis behind plant regenerative responses and key genes involved in these processes have been identified (Ikeuchi et al., 2019). BBM and WUS transcription factors are key regulators of cell totipotency. Both genes have been shown to induce somatic embryogenesis and organogenesis from vegetative cells when overexpressed (Bouchabké-Coussa et al.,

2013; Jha et al., 2020; Lowe et al., 2016). Studies involving hop transformation have focused on hormone regulated approaches for regenerating transgenic plants. Here we show that the transient expression of either *AtBBM* or *AtWUS* enhances competence of tissues undergoing organogenesis and improves transformation output in the hop genotype Galena (Table 2). We also show that regeneration can occur independent of exogenous cytokinin and auxin supplementation in the presence of DEX, the GR ligand. Plants transformed with either inducible transgene developed phenotypically normal.

Direct comparison of our *H35S.BBM* and *H35S.WUS* transformations with published attempts in hop is difficult as they involve the integration of either the reporter GUS or alternative transgenes; each of which may differ in genomic integration and stability. Furthermore, published protocols vary in their *Agrobacterium* use and were designed to be applicable to specific genotypes that differ in shoot-inducing hormone requirements. Two independent studies (Horlemann et al., 2003; Schwekendiek et al., 2005) using the same procedure for genetic transformation achieved overall efficiencies (input: internodal segments; output: transgenic plants growing in the greenhouse) of 1% and 0.2% in the transformation of the hop cultivar Tettnanger with GUS and stilbene synthase, respectively. Differences in efficiencies were hypothesized to be a consequence of secondary metabolite production or differential usage of the 35S promoter. Like Horlemann et al (2003), a separate study involving the transformation of the genotype Osvald-72 produced a final transformation efficiency of 1%, yet they were only able to regenerate a single stable transformant (Okada et al., 2003). Other reports failed to describe their final output (Mishra et al., 2018; Oriniakova & Matousek, 1996; Sousa et al., 1995).

We are still in the process of accurately determining the final number of transgenic plants per genetic transformation. As of currently, transformation efficiencies for *H35S.BBM* and *H35S.WUS* are based on the frequency of internodes that regenerate *Hpt* positive shoots (Table 2). However, as multiple shoots are generated per explant, our final efficiency has potential to far supersede what has been previously described, if stable transformation is successful. Following activation of either *AtBBM* or *AtWUS,* the percentage of explants regenerating shoots ranged from 1.2-1.7% for *H35S.BBM* and 0.34-0.5% for *H35S.WUS*, with 50-100% of primary shoots per explant testing positive for the T-DNA marker. No shoots were obtained on DEX-absent media, which was consistent with the lack of shoot regeneration in our *p35S.GUS*

transformations. This suggests that the scale of our experiments without the positive influence of *AtBBM* or *AtWUS* requires an increase in input to obtain shoots in the genotype Galena.

Although lower transformation efficiencies with *AtWUS* were achieved, it is difficult to determine if this was a result of the transgene itself or conditions of regeneration. *H35S.BBM* and *H35S.WUS* transformations were not conducted side-by-side and explants were sourced from different *in vitro* cultures. Internodes used for *H35S.WUS* were taken from *in vitro* plants that had experienced additional subculturing. A follow-up shoot regeneration test in wildtype Galena showed that regenerative potential of internodes declined when sampled from donor plants that were continuously propagated in nodal culture (every 4 weeks, >12 months) (data not shown). It is well known that regeneration potential can decline with increasing nodal culture age, but this generally occurs when shoots are not transferred to fresh medium in regular intervals (Barendse et al., 1985; Kathal et al., 1988; C. Liu, Callow, et al., 2010). In some species, regeneration capacity increases with additional subcultures (Liu, et al., 2010). The decline in hop regeneration efficiency in our experiments could be a consequence of somaclonal variation. Though this phenomenon is more common in callus cultures and exposure to 2,4-D (Bairu et al., 2011), epigenetic changes could occur following prolonged exposure to an *in vitro* environment. In addition, hop donor plants may require a period of dormancy to maintain their original regenerative potential. Hop produces annual shoots and recalcitrance to regenerative programs may increase if kept in a continued state of vegetative development in *in vitro*.

As previously stated, responsive explants in our *H35S.BBM* and *H35S.WUS* experiments regenerated more than one shoot (too many to count); however, current screening of *AtBBM* plants has indicated that shoots induced from the same explant are not all transgenic (Figure B.1). In some cases, shoots that originally tested positive, propagated future shoots that lacked T-DNA markers. Instability of transgenes and chimerism (only part of the tissue is transformed) has been reported in most published reports of hop transformation (Batista et al., 2008; Oriniakova et al., 1999; Schwekendiek et al., 2005; Škof & Luthar, 2005.). Škof and Luthar (2005) noted that the GUS reporter gene was lost in ~40% of shoots that originated from GUS positive regenerates. Furthermore only ~50% of transgenic shoots tested PCR-positive for both GUS and the antibiotic selection gene, suggesting incomplete integration of T-DNA. An

independent study conducted by Batista et al. (2008) observed both instability, chimerism and silencing of GUS. Interestingly, in addition to the loss of transgenes, they also identified GUS positive shoots via PCR that originated from plants that initially tested negative for the marker.

Accounts of chimerism and absence/loss of transgenes may be a consequence of the way that shoots are formed. Early inductive cues that initiate shoot regeneration begin with a group of cells (as opposed to a single cell event) that ultimately become the shoot primordia (Subban et al., 2021). This would account for the prevalence of chimeras if only few of the precursor cells are genetically transformed. In addition, low anti-biotic selection allows for the opportunity of untransformed tissue to gradually outgrow transgenic tissues, which would account for the loss of transgenes in shoots that were previously positive. Our current efforts are focusing on the genetic screening of *ex vitro* acclimated plants, followed by expression characterization of the *AtBBM* and *AtWUS* transgenes and their downstream targets.

4.1.4. Hygromycin exposure inhibits shoot development in hop

Hygromycin selection of transgenic tissue has proven to be difficult in hop. We found selection inhibited shoot growth and resulted in die-back of *Hyg* positive plants, even when used in low concentrations. Difficulty with selection has been noted in past transformation efforts and has prevented the regeneration of transgenic shoots when either kanamycin (Horlemann et al., 2003; Oriniakova et al., 1999; Škof & Luthar, 2005) or hygromycin (Batista et al., 2008) was used. Škof and Luthar (2005) avoided the use of antibiotic selection altogether and opted for early PCR-screening of potential transformants, as they observed explant death and failed to regenerate shoots in their preliminary experiments. Horlemann et al. (2003) chose early and low selection during shoot induction followed by PCR screening, while Batista et al. (2007) alternated between low selection and “no-selection” phases.

The trade-off with low and no-selection regimes is that your chance of regenerating false positives and chimeric plants increases; a consistent issue in published reports (see section 4.1.3). Moreover, these methods rely on extensive PCR-screening. False positives and chimerism can be avoided by completing multiple rounds of successive shoot regeneration, using potential transformants as the source tissue.

Subsequent regeneration/selection gradually dilutes untransformed cells and produces individuals with uniform and heritable integration of the transgene (G. Q. Chen, 2011). Alternatively, one could avoid hygromycin toxicity during the crucial and sensitive phase of shoot induction and instead, select for positive transformants during rooting, potentially using a higher dose of selection to avoid escape. Different tissue types and phases of development have been reported to exhibit different sensitivities to antibiotic selections (Batista et al., 2008a; Meng et al., 2007). We are currently unaware of any reports stating the use of herbicide resistance genes (phosphinothricin, glyphosate or imidazolines) for selecting transgenic hop; however, herbicide selection systems are similar to antibiotic selections, as they rely on the regeneration of single-transformed cells in a body of untransformed tissue, dying from induced toxicity.

A potentially attractive alternative approach is the use of positive selection. These systems provide transgenic cells with a metabolic advantage over non-transformed cells, of which are out-competed. Joersbo and Okkels (1996) developed a novel selection strategy for the regeneration and selection of tobacco by combining the use of the β-glucuronidase gene and an inactive cytokinin as a glucuronide derivative (benzyl-adenine N-3-glucuronide). Upon hydrolysis via GUS, active cytokinin is released, stimulating transformed cells to regenerate. In this system the GUS gene serves two purposes, both as a selectable agent and as a screenable marker gene. Alternatively, as hop regeneration occurs in the absence of PGRs with the help of *AtBBM/AtWUS*, one could incorporate these transgenes into regeneration and selection workflows. An approach not only for hop, but other species that experience antibiotic selection sensitivity.

4.2. Characterization of GA metabolism genes in hop

4.2.1. Gene identification and phylogenetic relationships of HlGA metabolism genes

In higher plants the GA biosynthesis pathway can be divided into early and late stages. The early stage involves the conversion of GGDP to GA_{12} in a series of reactions catalyzed by CPS, KS, KO and KAO enzymes, while the later stage results in the formation of C_{20}- and C_{19}-GAs via GA20 and GA3 oxidases. GA2ox is involved in GA deactivation and plays a key role in the regulation of bioactive GA content. In silico screening of the hop genome identified 20 candidate genes representing the seven core

enzymes involved in the GA biosynthesis pathway (Table 3). Similar to *Arabidopsis*, we found single genes encoding the *HlCPS* and *HlKS* enzymes (Sun et al., 1992; Yamaguchi et al., 1998). Although more than one hop homolog was identified for *KO* and *KAO*, it is unclear whether all identified homologs participate in the biosynthesis of GA (Yamaguchi, 2008). *Arabidopsis* has one copy of *KO* and two copies of *KAO* (Ogawa et al., 2003). The rice genome contains multiple *CPS*-like, *KS*-like, and *KO*-like genes, most of which are contiguously arranged (Sakamoto et al., 2004). However, mutant and expression analyses show that, like *Arabidopsis*, the early biosynthesis enzymes are represented by single genes, while remaining *CPS*-like, *KS*-like, and *KO*-like genes are also involved in the biosynthesis of diterpene phytoalexins (Sakamoto et al., 2004). Therefore, functional analyses are still required to confirm the participation of early biosynthesis candidates in the production of GA.

Gene duplication followed by selective pressure is believed to be an important precursor to the functional diversification of gene families (Panchy et al., 2016). Genetic diversification amongst the GAox enzymes is species dependent. For example, there are 16, 21, 17, 24 and 28 GA oxidase genes identified in *Arabidopsis*, rice, grapevine, soybean, and banana tree, respectively (J. Chen et al., 2016; Han & Zhu, 2011; He et al., 2019; Hedden, 2001). In comparison, we found 13 *GAox*-like gene candidates in the Cascade hop genome (PRJNA562558) including, five *HlGA20ox*, three *HlGA3ox*, and five *HlGA2ox.* A typical observation when comparing the GAox enzymes is the lesser degree of divergence amongst the GA3ox sequences (Han & Zhu, 2011), suggesting these enzymes are under greater selective pressure relative to the other oxidases. In contrast, GA20ox and GA2ox are generally quite diversified, resulting in greater functional redundancy. The presence of more GA20ox and GA2ox copies may allow for relaxed selective pressure or loosened constraints during their evolution (Han & Zhu, 2011), which could explain why we observed a greater degree of sequence divergence in both groups of enzymes. Sequence divergence may also be consequence of functional diversification (Han & Zhu, 2011). In contrast to early stages in GA biosynthesis, the GAox enzymes are differentially regulated by developmental and environmental cues in order to regulate bioactive GA levels (Hedden & Phillips, 2000). Furthermore, it is known that the GA20ox enzyme is capable of catalyzing multiple steps, with different substrates during GA biosynthesis (Hedden & Phillips, 2000).

Phylogenetic analysis of the GA biosynthesis enzymes showed distinct groupings representing each enzyme class in *Arabidopsis*, *Cannabis,* and hop, indicating genes are conserved amongst their homologs (Figure 13). As expected, each enzyme class shared similar functional domains and each sub-group, common motif compositions. Motifs in amino acid sequences have also been described in similar reports with jute (Honi et al., 2020), banana tree (Chen et al., 2016) and grape (He et al., 2019); however, how these motifs specify given roles in the biosynthesis of GA has yet to be determined. Subcellular localization predictions via Plant-mPLoc show that *HlCPS*, *HlKS*, *HlKO* are localized to the chloroplast, *HlKAO* the endoplasmic reticulum and *HlGAox* the cytoplasm, which is consistent with previous reports (Reviewed in Hedden & Sponsel, 2015). As in *Arabidopsis*, rice and soybean, four distinct sub-groups were recognized amongst the GA oxidase enzymes, representing each of the functional groups: GA20ox, GA3ox, C-20 GA2ox, and C-19 GA2ox (Figure 13B). GA oxidases of *Arabidopsis*, Cannabis and hop were generally found to be more similar to their respective homologs within each sub-group than they were to each other, suggesting GA oxidase expansion occurred early in the evolution of the protein family.

Interestingly, we did not identify any C-20 GA2ox enzymes within our dataset. C-20 GA2ox enzymes are responsible for the hydroxylation of C_{20} GA precursors, inhibiting their conversion to bioactive GAs (Lo et al., 2008). Furthermore, *HlGA20ox2, HlGA20ox5*, *HlGA3ox1* and *HlGA3ox3*, sequences appeared truncated at the 5’ end. It is possible that C-20 GA2ox genes have been lost during evolution and that an ancestral gene to *HlGA20ox2, HlGA20ox5* lost the 5’ end before duplications, possibly with no effect on gene function. Since C-20 GA2ox reduces the production of bioactive GA, these genes may have been selected against in hop, to obtain the extreme elongation typical of a liana. Another explanation for missing genes is that our reference genome is incomplete. For reference though, the Cascade genome used in our analyses is 4.31 Gb (Padgitt-Cobb et al., 2021), while the Teamaker genome is 2.7 Gb (Hill et al., 2017), indicating that we are using one of the best available resources.

4.2.2. HlGA20ox and HlGA3ox enzymes exhibit tissue-specificity

Like other plants, the hop *GA20ox* and *GA3ox* genes exhibited both broad expression and obvious tissue-specificity, suggesting diversification of physiological functions across this group of proteins (Figure 14, 15). All *HlGAox* candidates, except

HlGA20ox3, were detected in roots, cones, apical and basal internodes, and immature and mature leaves. However, their relative expression varied for each tissue type. Amongst the candidates, higher expression was observed in younger tissues as opposed to those that have matured. It has been reported in a variety of models that tissues undergoing growth, generally have the highest *GAox* expression and contain the greatest amount of bioactive GA (Silverstone et al., 1997; V. A. Smith et al., 1992). Interestingly, we did detect GAox activity in basal internodes and mature leaves. As samples for our analysis were taken from hop plants that had yet to meet their full height potential, these tissues could still be experiencing a degree of maturation. Furthermore, preliminary experiments in our laboratory have demonstrated that GA may function in the development of gelatinous fibers in the stem of hop.

In *Arabidopsis*, *AtGA20ox1* is the dominant oxidase expressed in the internodes and loss-of-function produces a semi-dwarf phenotype with normal flower development. *AtGA20ox2* is also expressed in the stem; however, single mutants fail to significantly reduce stature due to genetic redundancy (Rieu et al., 2008). Interestingly, when both *AtGA20ox1* and *AtGA20ox2* gene functions are lost, a greater dwarfing effect is evident compared to the *Arabidopsis* single *ga20ox1* mutant (Rieu et al., 2008). Based on sequence comparison *HlGA20ox1* and *HlGA20ox2* clustered with the semi-dwarfing *Arabidopsis* genes. Furthermore, these two hop candidates showed the highest relative expression in elongating internodes, yet exhibited low expression in cones, hence why they were chosen for future loss of function analyses. *HlGA20ox4* was the dominant transcript detected in cones, suggesting this gene is of great importance to flower development in hops. As hop plants are typically harvested for their cones, it would be interesting to see if a secondary copy of the endogenous *HlGA20ox4* gene is capable of increasing cone size and number.

Amongst the GA3ox enzymes, *HlGA3ox2* was the dominant transcript in all tested tissue-types, indicating a dominant role in promoting bioactive GA content. In contrast, *HlGA3ox1* and *HlGA3ox3* exhibited relatively weak expression. This is consistent with expression analyses conducted in banana tree, where out of the four *GA3ox* genes identified, two appeared to be functionally dominant (Chen et al., 2016). *HlGA3ox2* showed the highest sequence similarity with *AtGA3ox1* and *AtGA3ox2*. *AtGA3ox1*, also known as GA4, is responsible for bioactive GAs during vegetative growth and like *AtGA20ox1*, produces a semi-dwarf phenotype without pleiotropic effects

(Hu et al., 2008; Mitchum et al., 2006). However, as the hop homolog was expressed relatively high in the cones, we decided to temporally avoid this target for CRISPR-induced mutagenesis.

As the role of *HlGA20ox* and *HlGA3ox* genes were evaluated in plants experiencing ambient conditions, it would be interesting to further investigate roles under differing environmental stimulus. For example, both *AtGA3ox1* and *AtGA3ox2* are functionally redundant in imbibed seeds at 22°C; however, only *AtGA3ox1* is induced under cold treatment (Yamauchi et al., 2004). Additionally, differential expression of the *HlGAox* biosynthesis genes in the presence of exogenous GA and PB should be addressed to confirm the participation of identified candidates in GA biosynthesis as well as to elucidate roles of feedforward and feedback regulation in hop.

4.2.3. Current efforts towards CRISPR-targeted editing of HlGA20ox1 and HlGA20ox2

Spatial analyses of the *HlGA20ox* and *HlGA3ox*-like genes identified two ideal targets for CRISPR-induced mutagenesis towards semi-dwarfism with normal flower development. *HlGA20ox1* and *HlGA20ox2* were chosen for their dominant expression during internode elongation and relatively low expression in female cones. Though we have just begun transformation efforts with synthesized CRISPR constructs, we have already obtained nine independent hop lines, each with multiple potential transformants awaiting genotyping. To improve regeneration capacity, we introduced the *35S::AtBBM:GR* expression cassette into a set of CRISPR vectors. Consistent with our previous findings, transformations with *AtBBM* activity improved transformation output, providing circumstantial evidence that *AtBBM* positively influences shoot regeneration in hop. Furthermore, the number of shoots obtained per explant significantly improved, implying *AtBBM* activity may provide a form of positive selection. Interestingly, CRISPR transformations with *AtBBM* activity appeared to regenerate shoots both indirectly and directly, suggesting two different pathways of regeneration were being utilized.

To date there has only been a single publication demonstrating the use of CRISPR in hop. Awasthi et al. (2021) targeted the visible endogenous marker gene *phytoene desaturase* (*PDS*) involved in carotenoid biosynthesis as proof-of-concept. Only 33.3% of transgenic regenerates were successfully edited at the target site, of which the expected albino phenotype was observed. As in previous transformation

accounts in hop, chimerism was identified in 20% of transformants. Preliminary PCR-screening of shoots obtained from one of our early transformation lines showed 28% of regenerates were transgenic; however, no insertion-deletion mutations were detected within the *GA20ox* target. Though we used the same CRISPR vector (minus *35S::AtBBM:GR*) as Awasthi et al. (2021), their study involved the introduction of a secondary gRNA expression cassette allowing for CRISPR-targeting at two different sites in the *PDS* gene simultaneously, improving the probability of a loss-of-function mutation. The use of two or more gRNAs targeting a single gene has proven to be more effective for achieving site-directed mutagenesis (Liu et al., 2019); however, implementation of a single gRNA reduces the risk of off-target effects.

Although we are too early on in our experiments to make statements regarding transformation and mutation frequencies, the lack of detectable deletions in our initial CRISPR regenerates could be a consequence of several factors. Expression of the *Cas9* and gRNA depends on both the T-DNA integration site (Jensen et al., 2017; Tamura et al., 2016) and the copy number of the transgene (Hobbs et al., 1993). Furthermore, the use of multiple 35S promoters to drive both the expression of *Cas9*, *Hpt* and *AtBBM* may be subject to silencing. 35S promoter silencing has already been suggested by two independent studies in hop with single promoter use (Batista et al., 2008a; Horlemann et al., 2003). A key factor determining the efficiency of CRISPR gene editing is the choice of gRNA. Experimental evidence suggests that the GC content of the guide sequence can influence mutation frequency, hence 30-80% GC is recommended (Liang et al., 2016). As the gRNA functions by interacting with Cas9, it is also suggested that the secondary structure can interfere with Cas9-gRNA-DNA complex formation and DNA cleavage by Cas9 (Jensen et al., 2017; Ma et al., 2015; Nishimasu et al., 2014). As the spacer sequence of the gRNA is interchangeable, the final structure of the gRNA will vary. Hence why it is important to assess more than one gRNA when conducting experiments. We are currently in the process of evaluating the efficiency of two different gRNAs per *GA20ox* target, with the intention of expanding.

4.3. Concluding remarks

Compared to monoecious species, breeding in dioecious hop is relatively underdeveloped. This is in part due to the difficulty in generating mutants with homozygous recessive alleles. Recently developed CRISPR technology can potentially

overcome this limitation, as it is can be used to generate homozygous mutants in targeted genes in the first generation. This project aimed at taking the first steps at CRISPR-targeted breeding in hop.

As successful transformation in hop is negatively influenced by regeneration recalcitrance, this project focused greatly on obtaining and improving shoot regeneration. Here we attempted three different approaches for optimization: hormone induction, chemical enhancement and ectopic morphogenic gene expression. Transient expression of *AtBBM* and *AtWUS* were able to promote shoot regeneration in the absence of PGRs, showing promise for overcoming genotype-specific hormone requirements in hop. Future work should address the use of *AtBBM* and *AtWUS* transgenes in additional cultivars as well as different explant sources. Two strategies can be implemented using *AtBBM/AtWUS*-based transformation tools: (1) *BBM/WUS* plants can be used as an explant source for the introduction of a second gene of interest or (2) a second gene of interest can be introduced along with the *AtBBM/AtWUS* expression cassette in a single transformation event, as we have demonstrated with our CRISPR constructs.

Semi-dwarfism is a valuable and widely used trait in agriculture. Increased yield, pest resistance, lodging resistance, ease of harvest, are all attributes that have been used to describe semi-dwarfed genotypes. Successful manipulation of hop stature with GA and PB applications confirm that GA biosynthesis is a suitable target for genetic manipulation. Moreover, our expression analyses show that identified *HlGA20ox1* and *HlGA20ox2* are ideal candidates for targeted CRISPR-induced mutagenesis towards a semi-dwarf phenotype with normal flower development. Genotyping of potential transgenic hop plants is currently underway. Once the ideal phenotype is achieved, parallel efforts will focus on obtaining plants that lack the T-DNA insertion via traditional crossing to segregate the mutation from the T-DNA in order to obtain transgene free individuals.

References

Abram, V., Čeh, B., Vidmar, M., Hercezi, M., Lazić, N., Bucik, V., Možina, S. S., Košir, I. J., Kač, M., Demšar, L., & Poklar Ulrih, N. (2015). A comparison of antioxidant and antimicrobial activity between hop leaves and hop cones. *Industrial Crops and Products*, *64*, 124–134.

Ahmad, N., & Faisal, M. (Eds.). (2018). *Thidiazuron: From Urea Derivative to Plant Growth Regulator*. Springer Singapore.

Aida, M., Beis, D., Heidstra, R., Willemsen, V., Blilou, I., Galinha, C., Nussaume, L., Noh, Y.-S., Amasino, R., & Scheres, B. (2004). The PLETHORA Genes Mediate Patterning of the *Arabidopsis* Root Stem Cell Niche. *Cell*, *119*(1), 109–120.

Aida, M., Ishida, T., & Tasaka, M. (1999). Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: Interaction among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. *Development*, *126*(8), 1563–1570.

Almaguer, C., Schönberger, C., Gastl, M., Arendt, E. K., & Becker, T. (2014). *Humulus lupulus* – a story that begs to be told. A review. *Journal of the Institute of Brewing*, *120*(4), 289–314.

Aloni, R. (2010). The Induction of Vascular Tissues by Auxin. In P. J. Davies (Ed.), *Plant Hormones: Biosynthesis, Signal Transduction, Action!* (pp. 485–518). Springer Netherlands.

Atta, R., Laurens, L., Boucheron-Dubuisson, E., Guivarc'h, A., Carnero, E., Giraudat-Pautot, V., Rech, P., & Chriqui, D. (2009). Pluripotency of *Arabidopsis* xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown in vitro. *The Plant Journal*, *57*(4), 626–644.

Awasthi, P., Kocábek, T., Mishra, A. K., Nath, V. S., Shrestha, A., & Matoušek, J. (2021). Establishment of CRISPR/Cas9 mediated targeted mutagenesis in hop (*Humulus lupulus*). *Plant Physiology and Biochemistry*, *160*, 1–7.

Bairu, M. W., Aremu, A. O., & Van Staden, J. (2011). Somaclonal variation in plants: Causes and detection methods. *Plant Growth Regulation*, *63*(2), 147–173.

Banno, H., Ikeda, Y., Niu, Q.-W., & Chua, N.-H. (2001). Overexpression of *Arabidopsis* ESR1 Induces Initiation of Shoot Regeneration. *The Plant Cell*, *13*(12), 2609–2618.

Barendse, G. W. M., Ceoes, A. F., van den Ende, G., Bosveld, M., & Creemers, T. (1985). Role of hormones on flower bud formation in thin-layer expiants of tobacco. *Biologia Plantarum*, *27*(4–5), 408–412.

Bassil, N. V., Gilmore, B., Oliphant, J. M., Hummer, K. E., & Henning, J. A. (2008). Genic SSRs for European and North American hop (*Humulus lupulus L.*). *Genetic Resources and Crop Evolution*, *55*(7), 959–969.

Batista, D., Fonseca, S., Serrazina, S., Figueiredo, A., & Pais, M. S. (2008a). Efficient and stable transformation of hop (*Humulus lupulus L.*) var. Eroica by particle bombardment. *Plant Cell Reports*, *27*(7), 1185–1196.

Batista, D., Fonseca, S., Serrazina, S., Figueiredo, A., & Pais, M. S. (2008b). Efficient and stable transformation of hop (*Humulus lupulus L.*) var. Eroica by particle bombardment. *Plant Cell Reports*, *27*(7), 1185–1196.

Batista, D., Sousa, M. J., & Pais, M. S. (1996). Plant regeneration from stem and petiole-derived callus of *Humulus lupulus L.* (hop) clone Bragança and var. Brewer's Gold. *In Vitro – Plant*, *32*(1), 37–41.

Beatson, R. A., Ansell, K. A., & Graham, L. T. (2003). Breeding, development, and characteristics of the hop (*Humulus lupulus*) cultivar 'Nelson Sauvin.' *New Zealand Journal of Crop and Horticultural Science*, *31*(4), 303–309.

Benayoun, J., Aloni, R., & Sachs, T. (1975). Regeneration Around Wounds and the Control of Vascular Differentiation. *Annals of Botany*, *39*(3), 447–454.

Benkherouf, A. Y., Eerola, K., Soini, S. L., & Uusi-Oukari, M. (2020). Humulone Modulation of GABAA Receptors and Its Role in Hops Sleep-Promoting Activity. *Frontiers in Neuroscience*, *14*, 1100.

Bhatia, P., Ashwath, N., & Midmore, D. J. (2005). Effects of Genotype, Explant Orientation, and Wounding on Shoot Regeneration in Tomato. *In Vitro Cellular & Developmental Biology. Plant*, *41*(4), 457–464.

Bhuvaneswari, S., Gopala Krishnan, S., Ellur, R. K., Vinod, K. K., Bollinedi, H., Bhowmick, P. K., Bansal, V. P., Nagarajan, M., & Singh, A. K. (2020). Discovery of a Novel Induced Polymorphism in SD1 Gene Governing Semi-Dwarfism in Rice and Development of a Functional Marker for Marker-Assisted Selection. *Plants*, *9*(9), 1198.

Bilyeu, K. D., Cole, J. L., Laskey, J. G., Riekhof, W. R., Esparza, T. J., Kramer, M. D., & Morris, R. O. (2001). Molecular and Biochemical Characterization of a Cytokinin Oxidase from Maize. *Plant Physiology*, *125*(1), 378–386.

Bishopp, A., Lehesranta, S., Vatén, A., Help, H., El-Showk, S., Scheres, B., Helariutta, K., Mähönen, A. P., Sakakibara, H., & Helariutta, Y. (2011). Phloem-Transported Cytokinin Regulates Polar Auxin Transport and Maintains Vascular Pattern in the Root Meristem. *Current Biology*, *21*(11), 927–932.

Boboc Oros, P., Cantor, M., Cordea, M. I., & Cătană, C. (2022). Plant Regeneration Protocol for Recalcitrant Passionflower (*Passiflora quadrangularis L.*). *Horticulturae*, *8*(4), 337.

Boonkaew, T., Mongkolsiriwatana, C., Vongvanrungruang, A., Srikulnath, K., & Peyachoknagul, S. (2018). Characterization of GA20ox genes in tall and dwarf types coconut (*Cocos nucifera L.*). *Genes & Genomics*, *40*(7), 735–745.

Bouchabké-Coussa, O., Obellianne, M., Linderme, D., Montes, E., Maia-Grondard, A., Vilaine, F., & Pannetier, C. (2013). *Wuschel* overexpression promotes somatic embryogenesis and induces organogenesis in cotton (*Gossypium hirsutum L.*) tissues cultured in vitro. *Plant Cell Reports*, *32*(5), 675–686.

Boutilier, K., Offringa, R., Sharma, V. K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Liu, C.-M., van Lammeren, A. A. M., Miki, B. L. A., Custers, J. B. M., & van Lookeren Campagne, M. M. (2002). Ectopic Expression of *BABY BOOM* Triggers a Conversion from Vegetative to Embryonic Growth. *The Plant Cell*, *14*(8), 1737–

Brand, U., Fletcher, J. C., Hobe, M., Meyerowitz, E. M., & Simon, R. (2000). Dependence of Stem Cell Fate in *Arabidopsis* on a Feedback Loop Regulated by CLV3 Activity. *Science*, *289*(5479), 617–619.

Buechel, S., Leibfried, A., To, J. P. C., Zhao, Z., Andersen, S. U., Kieber, J. J., & Lohmann, J. U. (2010). Role of A-type *ARABIDOPSIS RESPONSE REGULATORS* in meristem maintenance and regeneration. *European Journal of Cell Biology*, *89*(2), 279–284. https://doi.org/10.1016/j.ejcb.2009.11.016

Bui, L. T., Pandzic, D., Youngstrom, C. E., Wallace, S., Irish, E. E., Szövényi, P., & Cheng, C.-L. (2017). A fern *AINTEGUMENTA* gene mirrors *BABY BOOM* in promoting apogamy in *Ceratopteris richardii*. *The Plant Journal*, *90*(1), 122–132.

Cancino, G. O., Gill, M. I., Anthony, P., Davey, M. R., Power, J. B., & Lowe, K. C. (2001). Pluronic F-68 enhanced shoot regeneration in a potentially novel citrus rootstock. *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*, *29*(4), 317–324.

Carrera, E., Jackson, S. D., & Prat, S. (1999). Feedback Control and Diurnal Regulation of Gibberellin 20-Oxidase Transcript Levels in Potato1. *Plant Physiology*, *119*(2), 765–774. https://doi.org/10.1104/pp.119.2.765

Cawrse, N. W., de Pomerai, D. I., & Lowe, K. C. (1991). Effects of Pluronic F-68 on 2-deoxyglucose uptake and amino acid incorporation into chick embryonic fibroblasts in vitro. *Biomedical Science*, *2*(2), 180–182.

Che, P., Lall, S., & Howell, S. H. (2007). Developmental steps in acquiring competence for shoot development in *Arabidopsis* tissue culture. *Planta*, *226*(5), 1183–1194.

Che, P., Lall, S., Nettleton, D., & Howell, S. H. (2006). Gene Expression Programs during Shoot, Root, and Callus Development in *Arabidopsis* Tissue Culture. *Plant Physiology*, *141*(2), 620–637.

Chen, G. Q. (2011). Effective reduction of chimeric tissue in transgenics for the stable genetic transformation of *Lesquerella fendleri*. *HortScience*, *46*(1), 86–90.

Chen, J., Xie, J., Duan, Y., Hu, H., Hu, Y., & Li, W. (2016). Genome-wide identification and expression profiling reveal tissue-specific expression and differentially-regulated genes involved in gibberellin metabolism between Williams banana and its dwarf mutant. *BMC Plant Biology*, *16*(1), 123.

Chen, L., Tong, J., Xiao, L., Ruan, Y., Liu, J., Zeng, M., Huang, H., Wang, J.-W., & Xu, L. (2016). YUCCA-mediated auxin biogenesis is required for cell fate transition occurring during de novo root organogenesis in *Arabidopsis*. *Journal of Experimental Botany*, *67*(14), 4273–4284.

Cheng, Z. J., Wang, L., Sun, W., Zhang, Y., Zhou, C., Su, Y. H., Li, W., Sun, T. T., Zhao, X. Y., Li, X. G., Cheng, Y., Zhao, Y., Xie, Q., & Zhang, X. S. (2013). Pattern of Auxin and Cytokinin Responses for Shoot Meristem Induction Results from the Regulation of Cytokinin Biosynthesis by AUXIN RESPONSE FACTOR3. *Plant Physiology*, *161*(1), 240–251.

Cho, H. T., & Kende, H. (1997). Expression of expansin genes is correlated with growth in deepwater rice. *The Plant Cell*, *9*(9), 1661–1671.

Christianson, M. L., & Warnick, D. A. (1983). Competence and determination in the process of in vitro shoot organogenesis. *Developmental Biology*, *95*(2), 288–293.

Christopher, T., & Rajam, M. V. (1996). Effect of genotype, explant and medium onin vitro regeneration of red pepper. *Plant Cell, Tissue and Organ Culture*, *46*(3), 245–250.

Chugh, S., Guha, S., & Rao, I. U. (2009). Micropropagation of orchids: A review on the potential of different explants. *Scientia Horticulturae*, *122*(4), 507–520.

Ci, J., Wang, X., Wang, Q., Zhao, F., Yang, W., Cui, X., Jiang, L., Ren, X., & Yang, W. (2021). Genome-wide analysis of gibberellin-dioxygenases gene family and their responses to GA applications in maize. *PLOS ONE*, *16*(5), e0250349.

Ckurshumova, W., Smirnova, T., Marcos, D., Zayed, Y., & Berleth, T. (2014). Irrepressible MONOPTEROS/ARF5 promotes de novo shoot formation. *New Phytologist*, *204*(3), 556–566.

Coleman, G. D., & Ernst, S. G. (1989). In vitro shoot regeneration of *Populus deltoides*: Effect of cytokinin and genotype. *Plant Cell Reports*, *8*(8), 459–462.

Darby, P. (2007). The UK hop breeding programme: A new site and new objectives. *International Hop Growers' Convention, Scientific Commission, IHGC, Tettnang, Germany*, 10–13.

Debnath, C. S. (2006). Zeatin overcomes thidiazuron-induced inhibition of shoot elongation and promotes rooting in strawberry culture in vitro. *The Journal of Horticultural Science and Biotechnology*, *81*(3), 349–354.

Debnath, S. C. (2005). A Two-step Procedure for Adventitious Shoot Regeneration from in vitro-derived Lingonberry Leaves: Shoot Induction with TDZ and Shoot Elongation Using Zeatin. *HortScience*, *40*(1), 189–192.

Dewir, Y. H., Nurmansyah, Naidoo, Y., & Teixeira da Silva, J. A. (2018). Thidiazuron-induced abnormalities in plant tissue cultures. *Plant Cell Reports*, *37*(11), 1451–1470.

Elhiti, M., Stasolla, C., & Wang, A. (2013). Molecular regulation of plant somatic embryogenesis. *In Vitro Cellular & Developmental Biology - Plant*, *49*(6), 631–642.

Elliott, R. C., Ross, J. J., Smith, J. J., Lester, D. R., & Reid, J. B. (2001). Feed-Forward Regulation of Gibberellin Deactivation in Pea. *Journal of Plant Growth Regulation*, *20*(1), 87.

Fabian, T., Lorbiecke, R., Umeda, M., & Sauter, M. (2000). The cell cycle genes cycA1;1 and cdc2Os-3 are coordinately regulated by gibberellin in planta. *Planta*, *211*(3), 376–383.

Fan, M., Xu, C., Xu, K., & Hu, Y. (2012). LATERAL ORGAN BOUNDARIES DOMAIN transcription factors direct callus formation in *Arabidopsis* regeneration. *Cell Research*, *22*(7), 1169–1180.

Gaj, M. D., Zhang, S., Harada, J. J., & Lemaux, P. G. (2005). Leafy cotyledon genes are essential for induction of somatic embryogenesis of *Arabidopsis*. *Planta*, *222*(6), 977–988.

Gajdošová, S., Spíchal, L., Kamínek, M., Hoyerová, K., Novák, O., Dobrev, P. I., Galuszka, P., Klíma, P., Gaudinová, A., Žižková, E., Hanuš, J., Dančák, M., Trávníček, B., Pešek, B., Krupička, M., Vaňková, R., Strnad, M., & Motyka, V. (2011). Distribution, biological activities, metabolism, and the conceivable function of cis-zeatin-type cytokinins in plants. *Journal of Experimental Botany*, *62*(8), 2827–2840.

Galinha, C., Hofhuis, H., Luijten, M., Willemsen, V., Blilou, I., Heidstra, R., & Scheres, B. (2007). PLETHORA proteins as dose-dependent master regulators of *Arabidopsis* root development. *Nature*, *449*(7165), 1053–1057.

Gao, S., & Chu, C. (2020). Gibberellin Metabolism and Signaling: Targets for Improving Agronomic Performance of Crops. *Plant and Cell Physiology*, *61*(11), 1902–1911.

García-Fortea, E., Lluch-Ruiz, A., Pineda-Chaza, B. J., García-Pérez, A., Bracho-Gil, J. P., Plazas, M., Gramazio, P., Vilanova, S., Moreno, V., & Prohens, J. (2020). A highly efficient organogenesis protocol based on zeatin riboside for in vitro regeneration of eggplant. *BMC Plant Biology*, *20*(1), 6.

Gatica-Arias, A., & Weber, G. (2013). Genetic transformation of hop (*Humulus lupulus L.* cv. Tettnanger) by particle bombardment and plant regeneration using a temporary immersion system. *In Vitro Cellular & Developmental Biology - Plant*, *49*(6), 656–664.

Genkov, T., Ivanova, I., Genkov, T., & Ivanova, I. (1995). *EFFECT OF CYTOKININ-ACTIVE PHENYLUREA DERIVATIVES ON SHOOT MULTIPLICATION, PEROXIDASE AND SUPEROXIDE DISMUTASE ACTIVITIES OF in vitro CULTURED CARNATION*.

Gordon, S. P., Heisler, M. G., Reddy, G. V., Ohno, C., Das, P., & Meyerowitz, E. M. (2007). Pattern formation during de novo assembly of the *Arabidopsis* shoot meristem. *Development*, *134*(19), 3539–3548.

Gordon-Kamm, B., Sardesai, N., Arling, M., Lowe, K., Hoerster, G., Betts, S., & Jones, T. (2019). Using Morphogenic Genes to Improve Recovery and Regeneration of Transgenic Plants. *Plants*, *8*(2), 38.

Gurriarán, M. J., Revilla, M. A., & Tamés, R. S. (1999). Adventitious shoot regeneration in cultures of *Humulus lupulus L.* (hop) cvs. Brewers Gold and Nugget. *Plant Cell Reports*, *18*(12), 1007–1011.

Hall, A. J., Babish, J. G., Darland, G. K., Carroll, B. J., Konda, V. R., Lerman, R. H., Bland, J. S., & Tripp, M. L. (2008). Safety, efficacy and anti-inflammatory activity of rho iso-alpha-acids from hops. *Phytochemistry*, *69*(7), 1534–1547.

Han, F., & Zhu, B. (2011). Evolutionary analysis of three gibberellin oxidase genes in rice, *Arabidopsis*, and soybean. *Gene*, *473*(1), 23–35.

He, H., Liang, G., Lu, S., Wang, P., Liu, T., Ma, Z., Zuo, C., Sun, X., Chen, B., & Mao, J. (2019). Genome-Wide Identification and Expression Analysis of *GA2ox*, *GA3ox*, and *GA20ox* Are Related to Gibberellin Oxidase Genes in Grape (*Vitis vinifera L.*). *Genes*, *10*(9), 680.

Heale, J. B., Legg, T., & Connell, S. (1989). *Humulus lupulus L.* (Hop): In Vitro Culture; Attempted Production of Bittering Components and Novel Disease Resistance. In Y. P. S. Bajaj (Ed.), *Medicinal and Aromatic Plants II* (pp. 264–285). Springer.

Hecht, V., Vielle-Calzada, J.-P., Hartog, M. V., Schmidt, E. D. L., Boutilier, K., Grossniklaus, U., & de Vries, S. C. (2001). The *Arabidopsis Somatic Embryogenesis Receptor Kinase 1* Gene Is Expressed in Developing Ovules and Embryos and Enhances Embryogenic Competence in Culture. *Plant Physiology*, *127*(3), 803–816.

Hedden, P. (2001). Gibberellin Metabolism and Its Regulation. *Journal of Plant Growth Regulation*, *20*(4), 317–318.

Hedden, P. (2003). The genes of the Green Revolution. *Trends in Genetics*, *19*(1), 5–9.

Hedden, P., & Phillips, A. L. (2000). Gibberellin metabolism: New insights revealed by the genes. *Trends in Plant Science*, *5*(12), 523–530.

Hedden, P., & Sponsel, V. (2015a). A century of gibberellin research. *Journal of Plant Growth Regulation*, *34*(4), 740–760.

Hedden, P., & Sponsel, V. (2015b). A Century of Gibberellin Research. *Journal of Plant Growth Regulation*, *34*(4), 740–760.

Heidmann, I., De Lange, B., Lambalk, J., Angenent, G. C., & Boutilier, K. (2011). Efficient sweet pepper transformation mediated by the BABY BOOM transcription factor. *Plant Cell Reports*, *30*(6), 1107–1115.

Helliwell, C. A., Poole, A., James Peacock, W., & Dennis, E. S. (1999). *Arabidopsis* ent-Kaurene Oxidase Catalyzes Three Steps of Gibberellin Biosynthesis. *Plant Physiology*, *119*(2), 507–510.

Helliwell, C. A., Sullivan, J. A., Mould, R. M., Gray, J. C., Peacock, W. J., & Dennis, E. S. (2001). A plastid envelope location of *Arabidopsis* ent-kaurene oxidase links the plastid and endoplasmic reticulum steps of the gibberellin biosynthesis pathway. *The Plant Journal*, *28*(2), 201–208.

Higuchi, M., Pischke, M. S., Mähönen, A. P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., & Tabata, S. (2004). In planta functions of the Arabidopsis cytokinin receptor family. *Proceedings of the National Academy of Sciences*, *101*(23), 8821–8826.

Hill, S. T., Sudarsanam, R., Henning, J., & Hendrix, D. (2017). HopBase: A unified resource for *Humulus* genomics. *Database*, *2017*.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., & Sakakibara, H. (2008). Regulation of cytokinin biosynthesis, compartmentalization and translocation. *Journal of Experimental Botany*, *59*(1), 75–83.

Hobbs, S. L. A., Warkentin, T. D., & DeLong, C. M. O. (1993). Transgene copy number can be positively or negatively associated with transgene expression. *Plant Molecular Biology*, *21*(1), 17–26.

Honi, U., Amin, Md. R., Kabir, S. M. T., Bashar, K. K., Moniruzzaman, Md., Jahan, R., Jahan, S., Haque, Md. S., & Islam, S. (2020). Genome-wide identification, characterization and expression profiling of gibberellin metabolism genes in jute. *BMC Plant Biology*, *20*(1), 306.

Hopslist | The Home of Beer Hops. (n.d.). Hopslist. Retrieved November 26, 2021, from http://www.hopslist.com/

Horlemann, C., Schwekendiek, A., Höhnle, M., & Weber, G. (2003). Regeneration and *Agrobacterium*-mediated transformation of hop (*Humulus lupulus L.*). *Plant Cell Reports*, *22*(3), 210–217.

Horstman, A., Bemer, M., & Boutilier, K. (2017). A transcriptional view on somatic embryogenesis. *Regeneration*, *4*(4), 201–216.

Horstman, A., Li, M., Heidmann, I., Weemen, M., Chen, B., Muino, J. M., Angenent, G. C., & Boutilier, K. (2017). The BABY BOOM Transcription Factor Activates the LEC1-ABI3-FUS3-LEC2 Network to Induce Somatic Embryogenesis. *Plant Physiology*, *175*(2), 848–857.

Horstman, A., Willemsen, V., Boutilier, K., & Heidstra, R. (2014). AINTEGUMENTA-LIKE proteins: Hubs in a plethora of networks. *Trends in Plant Science*, *19*(3), 146–157.

Hu, J., Mitchum, M. G., Barnaby, N., Ayele, B. T., Ogawa, M., Nam, E., Lai, W.-C., Hanada, A., Alonso, J. M., Ecker, J. R., Swain, S. M., Yamaguchi, S., Kamiya, Y., & Sun, T. (2008). Potential Sites of Bioactive Gibberellin Production during Reproductive Growth in *Arabidopsis*. *The Plant Cell*, *20*(2), 320–336.

Huetteman, C. A., & Preece, J. E. (1993). Thidiazuron: A potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture*, *33*(2), 105–119.

Hunkova, J., Libiaková, G., & Gajdošová, A. (2016). Shoot proliferation ability of selected cultivars of Rubus spp. As influenced by genotype and cytokinin concentration. *Journal of Central European Agriculture*.

Ikeuchi, M., Favero, D. S., Sakamoto, Y., Iwase, A., Coleman, D., Rymen, B., & Sugimoto, K. (2019). Molecular mechanisms of plant regeneration. *Annual Review of Plant Biology*, *70*, 377–406.

Ikeuchi, M., Ogawa, Y., Iwase, A., & Sugimoto, K. (2016). Plant regeneration: Cellular origins and molecular mechanisms. *Development*, *143*(9), 1442–1451.

Irshad, M., He, B., Liu, S., Mitra, S., Debnath, B., Li, M., Rizwan, H. M., & Qiu, D. (2017). In vitro regeneration of *Abelmoschus esculentus* L. cv. Wufu: Influence of anti-browning additives on phenolic secretion and callus formation frequency in explants. *Horticulture, Environment, and Biotechnology*, *58*(5), 503–513.

Ishida, T., Watanabe, B., Mashiguchi, K., & Yamaguchi, S. (2022). Synthesis and structure–activity relationship of 16,17-modified gibberellin derivatives. *Phytochemistry Letters*, *49*, 162–166.

Isogai, S., Touno, K., & Shimomura, K. (2008). Gibberellic acid improved shoot multiplication in *Cephaelis ipecacuanha*. *In Vitro Cellular & Developmental Biology - Plant*, *44*(3), 216–220.

Itoh, H., Ueguchi-Tanaka, M., Sentoku, N., Kitano, H., Matsuoka, M., & Kobayashi, M. (2001). Cloning and functional analysis of two gibberellin 3β-hydroxylase genes that are differently expressed during the growth of rice. *Proceedings of the National Academy of Sciences*, *98*(15), 8909–8914.

Iwase, A., Harashima, H., Ikeuchi, M., Rymen, B., Ohnuma, M., Komaki, S., Morohashi, K., Kurata, T., Nakata, M., Ohme-Takagi, M., Grotewold, E., & Sugimoto, K. (2017). WIND1 Promotes Shoot Regeneration through Transcriptional Activation

of ENHANCER OF SHOOT REGENERATION1 in *Arabidopsis*. *The Plant Cell*, *29*(1), 54–69.

Jakse, J., Satovic, Z., & Javornik, B. (2004). Microsatellite variability among wild and cultivated hops (*Humulus lupulus* L.). *Genome*, *47*(5), 889–899.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter*, *5*(4), 387–405.

Jensen, K. T., Fløe, L., Petersen, T. S., Huang, J., Xu, F., Bolund, L., Luo, Y., & Lin, L. (2017). Chromatin accessibility and guide sequence secondary structure affect CRISPR-Cas9 gene editing efficiency. *FEBS Letters*, *591*(13), 1892–1901.

Jha, P., & Kumar, V. (2018). BABY BOOM (BBM): A candidate transcription factor gene in plant biotechnology. *Biotechnology Letters*, *40*(11), 1467–1475.

Jha, P., Ochatt, S. J., & Kumar, V. (2020). WUSCHEL: A master regulator in plant growth signaling. *Plant Cell Reports*, *39*(4), 431–444.

Ji, Z. L., & Wang, S. Y. (1988). Reduction of abscisic acid content and induction of sprouting in potato, *Solanum tuberosum* L., by thidiazuron. *Journal of Plant Growth Regulation*, *7*(1), 37–44.

Jia, H., McCarty, D. R., & Suzuki, M. (2013). Distinct Roles of LAFL Network Genes in Promoting the Embryonic Seedling Fate in the Absence of VAL Repression. *Plant Physiology*, *163*(3), 1293–1305.

Joersbo, M., & Okkels, F. T. (1996). A novel principle for selection of transgenic plant cells: Positive selection. *Plant Cell Reports*, *16*(3), 219–221.

Jürgens, G., Mayer, U., Ramon A., T. R., Berleth, T., & Miséra, S. (1991). Genetic analysis of pattern formation in the *Arabidopsis* embryo. *Development*, *113* (Supplement_1), 27–38.

Kaparakis, G., & Alderson, P. G. (2015). Enhancement of in vitro cell proliferation of pepper (Capsicum annuum L.) by Pluronic F-68, haemoglobin and

arabinogalactan proteins. *The Journal of Horticultural Science and Biotechnology*.

Kareem, A., Durgaprasad, K., Sugimoto, K., Du, Y., Pulianmackal, A. J., Trivedi, Z. B., Abhayadev, P. V., Pinon, V., Meyerowitz, E. M., Scheres, B., & Prasad, K. (2015). *PLETHORA* Genes Control Regeneration by a Two-Step Mechanism. *Current Biology*, *25*(8), 1017–1030.

Karim, R., Tan, Y. S., Singh, P., Khalid, N., & Harikrishna, J. A. (2018). Expression and DNA methylation of SERK, BBM, LEC2 and WUS genes in in vitro cultures of Boesenbergia rotunda (L.) Mansf. *Physiology and Molecular Biology of Plants*, *24*(5), 741–751.

Kasahara, H., Hanada, A., Kuzuyama, T., Takagi, M., Kamiya, Y., & Yamaguchi, S. (2002). Contribution of the Mevalonate and Methylerythritol Phosphate Pathways to the Biosynthesis of Gibberellins in *Arabidopsis*. *Journal of Biological Chemistry*, *277*(47), 45188–45194.

Kathal, R., Bhatnagar, S. P., & Bhojwani, S. S. (1988). Regeneration of plants from leaf explants of Cucumis melo cv. Pusa Sharbati. *Plant Cell Reports*, *7*(6), 449–451.

Khaliluev, M. R., Bogoutdinova, L. R., Baranova, G. B., Baranova, E. N., Kharchenko, P. N., & Dolgov, S. V. (2014). Influence of genotype, explant type, and component of culture medium on in vitro callus induction and shoot organogenesis of tomato (*Solanum lycopersicum* L.). *Biology Bulletin*, *41*(6), 512–521.

Khatun, A., Laouar, L., Davey, M. R., Power, J. B., Mulligan, B. J., & Lowe, K. C. (1993). Effects of Pluronic F-68 on shoot regeneration from cultured jute cotyledons and on growth of transformed roots. *Plant Cell, Tissue and Organ Culture*, *34*(2), 133–140.

Kieber, J. J., & Schaller, G. E. (2014). Cytokinins. *The Arabidopsis Book / American Society of Plant Biologists*, *12*, e0168.

Kieber, J. J., & Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development*, *145*(4).

Kim, Yang, Forner, Lohmann, Noh, & Noh. (2018). Epigenetic reprogramming by histone acetyltransferase HAG1/AtGCN5 is required for pluripotency acquisition in *Arabidopsis*. *The EMBO Journal*, *37*(20), e98726.

King, A. T., Davey, M. R., Mellor, I. R., Mulligan, B. J., & Lowe, K. C. (1991). Surfactant effects on yeast cells. *Enzyme and Microbial Technology*, *13*(2), 148–153.

Kok, A. D.-X., Mohd Yusoff, N. F., Sekeli, R., Wee, C.-Y., Lamasudin, D. U., Ong-Abdullah, J., & Lai, K.-S. (2021). Pluronic F-68 Improves Callus Proliferation of Recalcitrant Rice Cultivar via Enhanced Carbon and Nitrogen Metabolism and Nutrients Uptake. *Frontiers in Plant Science*, *12*.

Kong, J., Martin-Ortigosa, S., Finer, J., Orchard, N., Gunadi, A., Batts, L. A., Thakare, D., Rush, B., Schmitz, O., Stuiver, M., Olhoft, P., & Pacheco-Villalobos, D. (2020). Overexpression of the Transcription Factor GROWTH-REGULATING FACTOR5 Improves Transformation of Dicot and Monocot Species. *Frontiers in Plant Science*, *11*.

Korpelainen, H., & Pietiläinen, M. (2021). Hop (Humulus lupulus L.): Traditional and Present Use, and Future Potential. *Economic Botany*, 1–21.

Krizek, B. A. (2015). AINTEGUMENTA-LIKE genes have partly overlapping functions with AINTEGUMENTA but make distinct contributions to *Arabidopsis thaliana* flower development. *Journal of Experimental Botany*, *66*(15), 4537–4549.

Kudo, T., Kiba, T., & Sakakibara, H. (2010). Metabolism and Long-distance Translocation of Cytokinins. *Journal of Integrative Plant Biology*, *52*(1), 53–60.

Kumar, V., Laouar, L., Davey, M. R., Mulligan, B. J., & Lowe, K. C. (1991). Effects of Pluronic F-68 on callus growth and protoplast plating efficiency of Solanum dulcamara. *Plant Cell Reports*, *10*(1), 52–54.

Laplaze, L., Parizot, B., Baker, A., Ricaud, L., Martinière, A., Auguy, F., Franche, C., Nussaume, L., Bogusz, D., & Haseloff, J. (2005). GAL4-GFP enhancer trap lines for genetic manipulation of lateral root development in Arabidopsis thaliana. *Journal of Experimental Botany*, *56*(419), 2433–2442.

Lawit, S. J., Wych, H. M., Xu, D., Kundu, S., & Tomes, D. T. (2010). Maize DELLA Proteins dwarf plant8 and dwarf plant9 as Modulators of Plant Development. *Plant and Cell Physiology*, *51*(11), 1854–1868.

Lee, S.-Y., & Kim, D.-I. (2002). Stimulation of murine granulocyte macrophage-colony stimulating factor production by Pluronic F-68 and polyethylene glycol in transgenic *Nicotiana tabacum* cell culture. *Biotechnology Letters*, *24*(21), 1779–1783.

Leibfried, A., To, J. P. C., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J. J., & Lohmann, J. U. (2005). *WUSCHEL* controls meristem function by direct regulation of cytokinin-inducible response regulators. *Nature*, *438*(7071), 1172–1175.

Lester, D. R., Ross, J. J., Davies, P. J., & Reid, J. B. (1997). Mendel's stem length gene (Le) encodes a gibberellin 3 beta-hydroxylase. *The Plant Cell*, *9*(8), 1435–1443.

Letham, D. S. (1973). Cytokinins from *Zea mays*. *Phytochemistry*, *12*(10), 2445–2455.

Li, M. Z., & Elledge, S. J. (2007). Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nature Methods*, *4*(3), 251–256.

Liang, G., Zhang, H., Lou, D., & Yu, D. (2016). Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. *Scientific Reports*, *6*(1), 21451.

Ling, A. C. K., Yap, C. P., Shaib, J. M., & Vilasini, P. (n.d.). *Induction and morphogenesis of Phalaenopsis callus*. 6.

Liu, C., Callow, P., Rowland, L. J., Hancock, J. F., & Song, G. (2010). Adventitious shoot regeneration from leaf explants of southern highbush blueberry cultivars. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *103*(1), 137–144.

Liu, C., Wang, J., Huang, T., Wang, F., Yuan, F., Cheng, X., Zhang, Y., Shi, S., Wu, J., & Liu, K. (2010). A missense mutation in the VHYNP motif of a DELLA protein causes a semi-dwarf mutant phenotype in *Brassica napus*. *Theoretical and Applied Genetics*, *121*(2), 249–258.

Liu, H., Ma, X., Han, H. N., Hao, Y. J., & Zhang, X. S. (2016). AtPRMT5 Regulates Shoot Regeneration through Mediating Histone H4R3 Dimethylation on KRPs and Pre-mRNA Splicing of RKP in *Arabidopsis*. *Molecular Plant*, *9*(12), 1634–1646.

Liu, H., Zhang, H., Dong, Y. X., Hao, Y. J., & Zhang, X. S. (2018). DNA METHYLTRANSFERASE1-mediated shoot regeneration is regulated by cytokinin-induced cell cycle in *Arabidopsis*. *New Phytologist*, *217*(1), 219–232.

Liu, J., Sheng, L., Xu, Y., Li, J., Yang, Z., Huang, H., & Xu, L. (2014). WOX11 and 12 Are Involved in the First-Step Cell Fate Transition during de Novo Root Organogenesis in *Arabidopsis*. *The Plant Cell*, *26*(3), 1081–1093.

Liu, Y., Gao, Y., Gao, Y., & Zhang, Q. (2019). Targeted deletion of floral development genes in *Arabidopsis* with CRISPR/Cas9 using the RNA endoribonuclease Csy4 processing system. *Horticulture Research*, *6*, 99.

Lo, S.-F., Yang, S.-Y., Chen, K.-T., Hsing, Y.-I., Zeevaart, J. A. D., Chen, L.-J., & Yu, S.-M. (2008). A novel class of gibberellin 2-oxidases control semidwarfism, tillering, and root development in rice. *The Plant Cell*, *20*(10), 2603–2618.

Locascio, A., Blázquez, M. A., & Alabadí, D. (2013). Genomic Analysis of DELLA Protein Activity. *Plant and Cell Physiology*, *54*(8), 1229–1237.

Lorbiecke, R., & Sauter, M. (1998). Induction of cell growth and cell division in the intercalary meristem of submerged deepwater rice (*Oryza sativa* L.). *Planta*, *204*(2), 140–145.

Lotan, T., Ohto, M., Yee, K. M., West, M. A. L., Lo, R., Kwong, R. W., Yamagishi, K., Fischer, R. L., Goldberg, R. B., & Harada, J. J. (1998). *Arabidopsis* LEAFY COTYLEDON1 Is Sufficient to Induce Embryo Development in Vegetative Cells. *Cell*, *93*(7), 1195–1205.

Lowe, K., Wu, E., Wang, N., Hoerster, G., Hastings, C., Cho, M.-J., Scelonge, C., Lenderts, B., Chamberlin, M., Cushatt, J., Wang, L., Ryan, L., Khan, T., Chow-Yiu, J., Hua, W., Yu, M., Banh, J., Bao, Z., Brink, K., … Gordon-Kamm, W. (2016). Morphogenic Regulators Baby boom and Wuschel Improve Monocot Transformation. *The Plant Cell*, *28*(9), 1998–2015.

Lu, C.-Y. (1993). The use of thidiazuron in tissue culture. *In Vitro Cellular & Developmental Biology - Plant*, *29*(2), 92–96.

Luo, G., & Palmgren, M. (2020). GRF-GIF chimeras boost plant regeneration. *Trends in Plant Science*.

Lutz, K. A., Martin, C., Khairzada, S., & Maliga, P. (2015). Steroid-inducible BABY BOOM system for development of fertile *Arabidopsis thaliana* plants after prolonged tissue culture. *Plant Cell Reports*, *34*(10), 1849–1856.

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., Lin, Y., Xie, Y., Shen, R., Chen, S., Wang, Z., Chen, Y., Guo, J., Chen, L., Zhao, X., Dong, Z., & Liu, Y.-G. (2015). A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. *Molecular Plant*, *8*(8), 1274–1284.

MacMillan, J. (2001). Occurrence of Gibberellins in Vascular Plants, Fungi, and Bacteria. *Journal of Plant Growth Regulation*, *20*(4), 387–442.

Magome, H., Nomura, T., Hanada, A., Takeda-Kamiya, N., Ohnishi, T., Shinma, Y., Katsumata, T., Kawaide, H., Kamiya, Y., & Yamaguchi, S. (2013). CYP714B1 and CYP714B2 encode gibberellin 13-oxidases that reduce gibberellin activity in rice. *Proceedings of the National Academy of Sciences*, *110*(5), 1947–1952.

Magyar-Tábori, K., Dobránszki, J., Teixeira da Silva, J. A., Bulley, S. M., & Hudák, I. (2010). The role of cytokinins in shoot organogenesis in apple. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *101*(3), 251–267.

Maloukh, L., Matousek, J., Van Bockstaele, E., & Roldán-Ruiz, I. (2009). Housekeeping Gene Selection for Real Time-PCR Normalization in Female Hop (*Humulus lupulus* L) Tissues. *Journal of Plant Biochemistry and Biotechnology*, *18*(1), 53–58.

Matoušek, J., Kocábek, T., Patzak, J., Füssy, Z., Procházková, J., & Heyerick, A. (2012). Combinatorial analysis of lupulin gland transcription factors from R2R3Myb, bHLH and WDR families indicates a complex regulation of chs_H1 genes essential for prenylflavonoid biosynthesis in hop (*Humulus Lupulus* L.). *BMC Plant Biology*, *12*(1), 27.

Matsuo, N., Makino, M., & Banno, H. (2011). *Arabidopsis* ENHANCER OF SHOOT REGENERATION (ESR)1 and ESR2 regulate in vitro shoot regeneration and their expressions are differentially regulated. *Plant Science*, *181*(1), 39–46.

Mazur, E., Benková, E., & Friml, J. (2016). Vascular cambium regeneration and vessel formation in wounded inflorescence stems of *Arabidopsis*. *Scientific Reports*, *6*(1), 1–15.

Meier, S. J., Hatton, T. A., & Wang, D. I. C. (1999). Cell death from bursting bubbles: Role of cell attachment to rising bubbles in sparged reactors. *Biotechnology and Bioengineering*, *62*(4), 468–478.

Meng, Z.-H., Liang, A.-H., & Yang, W.-C. (2007). Effects of hygromycin on cotton cultures and its application in *Agrobacterium*-mediated cotton transformation. *In Vitro Cellular & Developmental Biology - Plant*, *43*(2), 111–118.

Merkle, S. A., Parrott, W. A., & Flinn, B. S. (1995). Morphogenic Aspects of Somatic Embryogenesis. In T. A. Thorpe (Ed.), *In Vitro Embryogenesis in Plants* (pp. 155–203). Springer Netherlands.

Milligan, S., Kalita, J., Pocock, V., Heyerick, A., De Cooman, L., Rong, H., & De Keukeleire, D. (2002). Oestrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin. *REPRODUCTION-CAMBRIDGE-*, *123*(2), 235–242.

Miranda, C. L., Stevens, J. F., Helmrich, A., Henderson, M. C., Rodriguez, R. J., Yang, Y.-H., Deinzer, M. L., Barnes, D. W., & Buhler, D. R. (1999). Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*Humulus lupulus*) in human cancer cell lines. *Food and Chemical Toxicology*, *37*(4), 271–285.

Mishra, A. K., Duraisamy, G. S., Khare, M., Kocábek, T., Jakse, J., Bříza, J., Patzak, J., Sano, T., & Matoušek, J. (2018). Genome-wide transcriptome profiling of transgenic hop (*Humulus lupulus* L.) constitutively overexpressing HlWRKY1 and HlWDR1 transcription factors. *BMC Genomics*, *19*(1), 739.

Mitchum, M. G., Yamaguchi, S., Hanada, A., Kuwahara, A., Yoshioka, Y., Kato, T., Tabata, S., Kamiya, Y., & Sun, T. (2006). Distinct and overlapping roles of two gibberellin 3-oxidases in *Arabidopsis* development. *The Plant Journal*, *45*(5), 804–818.

Mizobuchi, S., & Sato, Y. (1985). Antifungal activities of hop bitter resins and related compounds. *Agricultural and Biological Chemistry*, *49*(2), 399–403.

Mok, M. C., Mok, D. W. S., Armstrong, D. J., Rabakoarihanta, A., & Kim, S.-G. (1980). CYTOKININ AUTONOMY IN TISSUE CULTURES OF PHASEOLUS: A GENOTYPE-SPECIFIC AND HERITABLE TRAIT. *Genetics*, *94*(3), 675–686.

Monna, L., Kitazawa, N., Yoshino, R., Suzuki, J., Masuda, H., Maehara, Y., Tanji, M., Sato, M., Nasu, S., & Minobe, Y. (2002). Positional Cloning of Rice Semidwarfing Gene, *sd-1*: Rice "Green Revolution Gene" Encodes a Mutant Enzyme Involved in Gibberellin Synthesis. *DNA Research*, *9*(1), 11–17.

Morris, M. L., & Bellon, M. R. (2004). Participatory plant breeding research: Opportunities and challenges for the international crop improvement system. *Euphytica*, *136*(1), 21–35.

Moura, M., Candeias, M. I., & Silva, L. (2009). In Vitro Propagation of *Viburnum treleasei* Gand., an Azorean Endemic with High Ornamental Interest. *HortScience*, *44*(6), 1668–1671.

Mudunkothge, J. S., & Krizek, B. A. (2012). Three *Arabidopsis* AIL/PLT genes act in combination to regulate shoot apical meristem function. *The Plant Journal*, *71*(1), 108–121.

Murakami, A., Darby, P., Javornik, B., Pais, M. S. S., Seigner, E., Lutz, A., & Svoboda, P. (2006). Microsatellite DNA Analysis of Wild Hops, *Humulus lupulus L*. *Genetic Resources and Crop Evolution*, *53*(8), 1553–1562.

Murthy, B. N. S., Singh, R. P., & Saxena, P. K. (1996). Induction of high-frequency somatic embryogenesis in geranium (*Pelargonium* x *hortorum Bailey* cv Ringo Rose) cotyledonary cultures. *Plant Cell Reports*, *15*(6), 423–426.

Nakajima, M., Shimada, A., Takashi, Y., Kim, Y.-C., Park, S.-H., Ueguchi-Tanaka, M., Suzuki, H., Katoh, E., Iuchi, S., Kobayashi, M., Maeda, T., Matsuoka, M., & Yamaguchi, I. (2006). Identification and characterization of *Arabidopsis* gibberellin receptors. *The Plant Journal*, *46*(5), 880–889.

Negin, B., Shemer, O., Sorek, Y., & Williams, L. E. (2017). Shoot stem cell specification in roots by the WUSCHEL transcription factor. *PLOS ONE*, *12*(4), e0176093.

Neve, R. A. (2012). *Hops*. Springer Science & Business Media.

Nishimasu, H., Ran, F. A., Hsu, P. D., Konermann, S., Shehata, S. I., Dohmae, N., Ishitani, R., Zhang, F., & Nureki, O. (2014). Crystal Structure of Cas9 in Complex with Guide RNA and Target DNA. *Cell*, *156*(5), 935–949.

Ogas, J., Kaufmann, S., Henderson, J., & Somerville, C. (1999). PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *96*(24), 13839–13844.

Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A., Kamiya, Y., & Yamaguchi, S. (2003). Gibberellin Biosynthesis and Response during *Arabidopsis* Seed Germination. *The Plant Cell*, *15*(7), 1591–1604.

Okada, Y., Saeki, K., Inaba, A., Suda, N., Kaneko, T., & Ito, K. (2003). Construction of gene expression system in hop (*Humulus lupulus*) lupulin gland using valerophenone synthase promoter. *Journal of Plant Physiology*, *160*(9), 1101–1108.

Olszewski, N., Sun, T., & Gubler, F. (2002). Gibberellin Signaling: Biosynthesis, Catabolism, and Response Pathways. *The Plant Cell*, *14*(suppl_1), S61–S80. https://doi.org/10.1105/tpc.010476

O'Neill, D. P., & Ross, J. J. (2002). Auxin Regulation of the Gibberellin Pathway in Pea. *Plant Physiology*, *130*(4), 1974–1982. https://doi.org/10.1104/pp.010587

Oriniakova, P., & Matousek, J. (1996). Viroid infection of hop (*Humulus lupulus*) mediated by *Agrobacterium tumefaciens* and conditions for hop transformation. *Rostlinna Vyroba UZPI*.

Oriniakova, P., Pavingerova, D., & Matousek, J. (1999). Methodical aspects of hop (*Humulus lupulus*) genetic transformation. *Rostlinna Vyroba - UZPI (Czech Republic)*.

Padgitt-Cobb, L. K., Kingan, S. B., Wells, J., Elser, J., Kronmiller, B., Moore, D., Concepcion, G., Peluso, P., Rank, D., Jaiswal, P., Henning, J., & Hendrix, D. A. (2021). A draft phased assembly of the diploid Cascade hop (*Humulus lupulus*) genome. *The Plant Genome*, *14*(1), e20072.

Pai, S. R., & Desai, N. S. (2018). Effect of TDZ on Various Plant Cultures. In N. Ahmad & M. Faisal (Eds.), *Thidiazuron: From Urea Derivative to Plant Growth Regulator* (pp. 439–454).

Panchy, N., Lehti-Shiu, M., & Shiu, S.-H. (2016). Evolution of gene duplication in plants. *Plant Physiology*, *171*(4), 2294–2316.

Parismoreno Rivas, L., García-Caparrós, P., Llanderal, A., Teixeira da Silva, J., Reca, J., & Lao, M. (2019). Plant regeneration from nodal segments and protocorm-like bodies (PLBs) derived from Cattleya maxima J. Lindley in response to chitosan and coconut water. *PROPAGATION OF ORNAMENTAL PLANTS*, *19*, 18–23.

Passey, A. J., & Jones, O. P. (1983). Shoot proliferation and rooting in vitro of *Theobroma cacao* L. type Amelonado. *Journal of Horticultural Science*, *58*(4), 589–592.

Patzak, J., Nesvadba, V., Krofta, K., Henychova, A., Marzoev, A. I., & Richards, K. (2010). Evaluation of genetic variability of wild hops (*Humulus lupulus* L.) in Canada and the Caucasus region by chemical and molecular methods. *Genome*, *53*(7), 545–557.

Peixe, A., Raposo, A., Lourenço, R., Cardoso, H., & Macedo, E. (2007). Coconut water and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation. *Scientia Horticulturae*, *113*(1), 1–7.

Peng, J., Richards, D. E., Hartley, N. M., Murphy, G. P., Devos, K. M., Flintham, J. E., Beales, J., Fish, L. J., Worland, A. J., Pelica, F., Sudhakar, D., Christou, P.,

Snape, J. W., Gale, M. D., & Harberd, N. P. (1999). ‘Green revolution’ genes encode mutant gibberellin response modulators. *Nature*, *400*(6741), 256–261.

Phillips, A. L., Ward, D. A., Uknes, S., Appleford, NEJ., Lange, T., Huttly, A. K., Gaskin, P., Graebe, J. E., & Hedden, P. (1995). Isolation and Expression of Three Gibberellin 20-Oxidase cDNA Clones from *Arabidopsis*. *Plant Physiology*, *108*(3), 1049–1057.

Przemeck, G. K. H., Mattsson, J., Hardtke, C. S., Sung, Z. R., & Berleth, T. (1996). Studies on the role of the *Arabidopsis* gene *MONOPTEROS* in vascular development and plant cell axialization. *Planta*, *200*(2), 229–237.

Qiao, M., Zhao, Z., Song, Y., Liu, Z., Cao, L., Yu, Y., Li, S., & Xiang, F. (2012). Proper regeneration from in vitro cultured *Arabidopsis thaliana* requires the microRNA-directed action of an auxin response factor. *The Plant Journal*, *71*(1), 14–22.

Rajakani, R., Narnoliya, L., Sangwan, N. S., Sangwan, R. S., & Gupta, V. (2013). Activated charcoal-mediated RNA extraction method for *Azadirachta indica* and plants highly rich in polyphenolics, polysaccharides and other complex secondary compounds. *BMC Research Notes*, *6*(1), 125.

Rani, D., & Dantu, P. K. (2016). Sustained Shoot Multiplication and Method for Overcoming In Vitro Browning in Medicinally Important Plant, Piper chaba Hunt. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *86*(2), 407–413.

Regnault, T., Davière, J.-M., Heintz, D., Lange, T., & Achard, P. (2014). The gibberellin biosynthetic genes AtKAO1 and AtKAO2 have overlapping roles throughout *Arabidopsis* development. *The Plant Journal*, *80*(3), 462–474.

Rieu, I., Ruiz-Rivero, O., Fernandez-Garcia, N., Griffiths, J., Powers, S. J., Gong, F., Linhartova, T., Eriksson, S., Nilsson, O., Thomas, S. G., Phillips, A. L., & Hedden, P. (2008). The gibberellin biosynthetic genes *AtGA20ox1* and

AtGA20ox2 act, partially redundantly, to promote growth and development throughout the *Arabidopsis* life cycle. *The Plant Journal*, *53*(3), 488–504.

Roh, H. S., & Kim, J. B. (2014). Establishment of proliferation and regeneration system of PLBs in *Phalaenopsis* by treatments of a variety of types of medium, sucrose concentrations and anti-browning agents. *Journal of Plant Biotechnology*, *41*(4), 223–228.

Ross, J. J., O'neill, D. P., Smith, J. J., Kerckhoffs, L. H. J., & Elliott, R. C. (2000). Evidence that auxin promotes gibberellin A1 biosynthesis in pea. *The Plant Journal*, *21*(6), 547–552.

Ross, J. J., Reid, J. B., Gaskin, P., & MacMillan, J. (1989). Internode length in *Pisum*. Estimation of GA1 levels in genotypes Le, le and led. *Physiologia Plantarum*, *76*(2), 173–176.

Rosspopoff, O., Chelysheva, L., Saffar, J., Lecorgne, L., Gey, D., Caillieux, E., Colot, V., Roudier, F., Hilson, P., Berthomé, R., Da Costa, M., & Rech, P. (2017). Direct conversion of root primordium into shoot meristem relies on timing of stem cell niche development. *Development*, *144*(7), 1187–1200.

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., & Oka, A. (2001). ARR1, a Transcription Factor for Genes Immediately Responsive to Cytokinins. *Science*, *294*(5546), 1519–1521.

Sakamoto, T., Miura, K., Itoh, H., Tatsumi, T., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Agrawal, G. K., Takeda, S., Abe, K., Miyao, A., Hirochika, H., Kitano, H., Ashikari, M., & Matsuoka, M. (2004). An Overview of Gibberellin Metabolism Enzyme Genes and Their Related Mutants in Rice. *Plant Physiology*, *134*(4), 1642–1653.

Sasaki, A., Ashikari, M., Ueguchi-Tanaka, M., Itoh, H., Nishimura, A., Swapan, D., Ishiyama, K., Saito, T., Kobayashi, M., & Khush, G. S. (2002). A mutant gibberellin-synthesis gene in rice. *Nature*, *416*(6882), 701–702.

Savatin, D. V., Gramegna, G., Modesti, V., & Cervone, F. (2014). Wounding in the plant tissue: The defense of a dangerous passage. *Frontiers in Plant Science*, *5*, 470.

Schneider, G., Jensen, E., Spray, C. R., & Phinney, B. O. (1992). Hydrolysis and reconjugation of gibberellin A20 glucosyl ester by seedlings of *Zea mays* L. *Proceedings of the National Academy of Sciences*, *89*(17), 8045–8048.

Schomburg, F. M., Bizzell, C. M., Lee, D. J., Zeevaart, J. A. D., & Amasino, R. M. (2003). Overexpression of a Novel Class of Gibberellin 2-Oxidases Decreases Gibberellin Levels and Creates Dwarf Plants. *The Plant Cell*, *15*(1), 151–163.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K. F. X., Jürgens, G., & Laux, T. (2000). The Stem Cell Population of *Arabidopsis* Shoot Meristems Is Maintained by a Regulatory Loop between the *CLAVATA* and *WUSCHEL* Genes. *Cell*, *100*(6), 635–644.

Schwekendiek, A., Horlemann, C., Spring, O., Stanke, M., Höhnle, M., & Weber, G. (2005). Hop (*Humulus lupulus L.*) Transformation with stilbene synthase for increasing resistance against fungal pathogens. *Acta Horticulturae*, *668*, 101–108.

Shao, X., Wu, S., Dou, T., Zhu, H., Hu, C., Huo, H., He, W., Deng, G., Sheng, O., Bi, F., Gao, H., Dong, T., Li, C., Yang, Q., & Yi, G. (2020). Using CRISPR/Cas9 genome editing system to create *MaGA20ox2* gene-modified semi-dwarf banana. *Plant Biotechnology Journal*, *18*(1), 17–19.

Silverstone, A. L., Chang, C., Krol, E., & Sun, T. (1997). Developmental regulation of the gibberellin biosynthetic gene GA1 in *Arabidopsis thaliana*. *The Plant Journal*, *12*(1), 9–19.

Singh, C. K., Raj, S. R., Jaiswal, P. S., Patil, V. R., Punwar, B. S., Chavda, J. C., & Subhash, N. (2016). Effect of plant growth regulators on in vitro plant regeneration of sandalwood (*Santalum album* L.) via organogenesis. *Agroforestry Systems*, *90*(2), 281–288.

Sivanesan, I., & Park, S. W. (2014). The role of silicon in plant tissue culture. *Frontiers in Plant Science*, *5*. https://www.frontiersin.org/article/10.3389/fpls.2014.00571

Skof, S., Cerenak, A., Jakse, J., Bohanec, B., & Javornik, B. (2012). Ploidy and sex expression in monoecious hop (*Humulus lupulus*). *Botany*, *90*(7), 617–626.

Škof, S., & Luthar, Z. (n.d.). *Detection of the reporter and selection genes in transformed hop (Humulus lupulus L.)*. 8.

ŠKOF, S., & LUTHAR, Z. (2005). Detection of the reporter and selection genes in transformed hop (*Humulus lupulus* L.). *Acta Agricult. Sloven*, *85*, 351–358.

Skoog, F., & Miller, C. (1957). Chemical regulation of growth and organ formation in plant tissues cultured. *Vitro Symp Soc Exp Biol*.

Smith, R. C., & Fry, S. C. (1991). Endotransglycosylation of xyloglucans in plant cell suspension cultures. *Biochemical Journal*, *279*(2), 529–535.

Smith, R. C., Matthews, P. R., Schünmnn, P. H. D., & Chandler, P. M. (1996). The regulation of leaf elongation and xyloglucan endotransglycosylase by gibberellin in 'Himalaya' barley (*Hordeum vulgare* L.). *Journal of Experimental Botany*, *47*(9), 1395–1404.

Smith, V. A., Knatt, C. J., Gaskin, P., & Reid, J. B. (1992). The Distribution of Gibberellins in Vegetative Tissues of *Pisum sativum* L. 1: I. Biological and Biochemical Consequences of the le Mutation. *Plant Physiology*, *99*(2), 368–371.

Smýkalová, I., Ortová, M., Lipavská, H., & Patzak, J. (2001). Efficient In Vitro Micropropagation and Regeneration of *Humulus Lupulus* on Low Sugar, Starch-Gelrite Media. *Biologia Plantarum*, *44*(1), 7–12.

Sousa, J., Batista, D., & Pais, M. S. (1995). Regeneration protocol in *Humulus lupulus* L. and study of the conditions for transformation mediated by *Agrobacterium tumefaciens*. *PROCEEDINGS OF CONGRESS-EUROPEAN BREWERY CONVENTION*, *25*(1), 135–135.

Spielmeyer, W., Ellis, M. H., & Chandler, P. M. (2002). *Semidwarf* (*sd-1*), "green revolution" rice, contains a defective gibberellin 20-oxidase gene. *Proceedings of the National Academy of Sciences*, *99*(13), 9043–9048.

Spielmeyer, W., Ellis, M., Robertson, M., Ali, S., Lenton, J. R., & Chandler, P. M. (2004). Isolation of gibberellin metabolic pathway genes from barley and comparative mapping in barley, wheat and rice. *Theoretical and Applied Genetics*, *109*(4), 847–855.

Sponsel, V. M., & Hedden, P. (2010). Gibberellin biosynthesis and inactivation. In *Plant Hormones* (pp. 63–94). Springer.

Steinitz, B., Barr, N., Tabib, Y., Vaknin, Y., & Bernstein, N. (2010). Control of in vitro rooting and plant development in *Corymbia maculata* by silver nitrate, silver thiosulfate and thiosulfate ion. *Plant Cell Reports*, *29*(11), 1315–1323.

Stone, S. L., Kwong, L. W., Yee, K. M., Pelletier, J., Lepiniec, L., Fischer, R. L., Goldberg, R. B., & Harada, J. J. (2001). *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. *Proceedings of the National Academy of Sciences*, *98*(20), 11806–11811.

Su, Y. H., Liu, Y. B., Bai, B., & Zhang, X. S. (2015). Establishment of embryonic shoot–root axis is involved in auxin and cytokinin response during *Arabidopsis* somatic embryogenesis. *Frontiers in Plant Science*, *5*.

Subban, P., Kutsher, Y., Evenor, D., Belausov, E., Zemach, H., Faigenboim, A., Bocobza, S., Timko, M. P., & Reuveni, M. (2021). Shoot Regeneration Is Not a Single Cell Event. *Plants*, *10*(1), 58.

Sugimoto, K., Jiao, Y., & Meyerowitz, E. M. (2010). *Arabidopsis* Regeneration from Multiple Tissues Occurs via a Root Development Pathway. *Developmental Cell*, *18*(3), 463–471.

Sun, Tp., Goodman, H. M., & Ausubel, F. M. (1992). Cloning the *Arabidopsis* GA1 Locus by Genomic Subtraction. *The Plant Cell*, *4*(2), 119–128.

Suttle, J. C. (1986). Cytokinin-Induced Ethylene Biosynthesis in Nonsenescing Cotton Leaves. *Plant Physiology*, *82*(4), 930–935.

Tamura, K., Kawabayashi, T., Shikanai, T., & Hara-Nishimura, I. (2016). Decreased Expression of a Gene Caused by a T-DNA Insertion in an Adjacent Gene in *Arabidopsis*. *PLoS ONE*, *11*(2), e0147911.

Thomas, J. C., & Katterman, F. R. (1986). Cytokinin Activity Induced by Thidiazuron. *Plant Physiology*, *81*(2), 681–683.

Thomas, S. G., Rieu, I., & Steber, C. M. (2005). Gibberellin Metabolism and Signaling. In G. Litwack (Ed.), *Vitamins & Hormones* (Vol. 72, pp. 289–338). Academic Press.

Townsend, M. S., & Henning, J. A. (2009). AFLP Discrimination of Native North American and Cultivated Hop. *Crop Science*, *49*(2), 600–607.

Toyomasu, T., Kawaide, H., Sekimoto, H., von Numers, C., Phillips, A. L., Hedden, P., & Kamiya, Y. (1997). Cloning and characterization of a cDNA encoding gibberellin 20-oxidase from rice (*Oryza sativa*) seedlings. *Physiologia Plantarum*, *99*(1), 111–118.

Turner, S. F., Benedict, C. A., Darby, H., Hoagland, L. A., Simonson, P., Sirrine, J. R., & Murphy, K. M. (2011). Challenges and Opportunities for Organic Hop Production in the United States. *Agronomy Journal*, *103*(6), 1645–1654.

Tyrrell, E., Archer, R., Skinner, G. A., Singh, K., Colston, K., & Driver, C. (2010). Structure elucidation and an investigation into the in vitro effects of hop acids on human cancer cells. *Phytochemistry Letters*, *3*(1), 17–23.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T., Hsing, Y. C., Kitano, H., Yamaguchi, I., & Matsuoka, M. (2005). GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature*, *437*(7059), 693–698.

US Hop Production Reaches Saturation Point, Says Hop Growers. (2018, February 8). Brewbound. https://www.brewbound.com/news/us-hop-production-reaches-saturation-point-says-hop-growers

USA Hops—Hop Growers of America. (n.d.). Retrieved July 14, 2022, from https://www.usahops.org/

Varbanova, M., Yamaguchi, S., Yang, Y., McKelvey, K., Hanada, A., Borochov, R., Yu, F., Jikumaru, Y., Ross, J., Cortes, D., Ma, C. J., Noel, J. P., Mander, L., Shulaev, V., Kamiya, Y., Rodermel, S., Weiss, D., & Pichersky, E. (2007). Methylation of Gibberellins by *Arabidopsis GAMT1* and *GAMT2*. *The Plant Cell*, *19*(1), 32–45.

Verdeil, J.-L., Alemanno, L., Niemenak, N., & Tranbarger, T. J. (2007). Pluripotent versus totipotent plant stem cells: Dependence versus autonomy? *Trends in Plant Science*, *12*(6), 245–252.

Weigel, D., & Glazebrook, J. (2006). Transformation of agrobacterium using electroporation. *CSH Protocols*, *2006*(7).

Wójcikowska, B., Jaskóła, K., Gąsiorek, P., Meus, M., Nowak, K., & Gaj, M. D. (2013). LEAFY COTYLEDON2 (LEC2) promotes embryogenic induction in somatic tissues of *Arabidopsis*, via YUCCA-mediated auxin biosynthesis. *Planta*, *238*(3), 425–440.

Wolbang, C. M., Chandler, P. M., Smith, J. J., & Ross, J. J. (2004). Auxin from the Developing Inflorescence Is Required for the Biosynthesis of Active Gibberellins in Barley Stems. *Plant Physiology*, *134*(2), 769–776.

Wolbang, C. M., & Ross, J. J. (2001). Auxin promotes gibberellin biosynthesis in decapitated tobacco plants. *Planta*, *214*(1), 153–157.

Wybouw, B., & De Rybel, B. (2019). Cytokinin – A Developing Story. *Trends in Plant Science*, *24*(2), 177–185.

Xin, Z., & Chen, J. (2012). A high throughput DNA extraction method with high yield and quality. *Plant Methods*, *8*(1), 26.

Xing, H.-L., Dong, L., Wang, Z.-P., Zhang, H.-Y., Han, C.-Y., Liu, B., Wang, X.-C., & Chen, Q.-J. (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology*, *14*, 327.

Xu, Y., Jia, Q., Zhou, G., Zhang, X.-Q., Angessa, T., Broughton, S., Yan, G., Zhang, W., & Li, C. (2017). Characterization of the *sdw1* semi-dwarf gene in barley. *BMC Plant Biology*, *17*(1), 11.

Yadav, R. K., Perales, M., Gruel, J., Girke, T., Jönsson, H., & Reddy, G. V. (2011). WUSCHEL protein movement mediates stem cell homeostasis in the *Arabidopsis* shoot apex. *Genes & Development*, *25*(19), 2025–2030.

Yamaguchi, S. (2008). Gibberellin metabolism and its regulation. *Annu. Rev. Plant Biol.*, *59*, 225–251.

Yamaguchi, S., Sun, T., Kawaide, H., & Kamiya, Y. (1998). The GA2 Locus of *Arabidopsis thaliana* Encodes ent-Kaurene Synthase of Gibberellin Biosynthesis. *Plant Physiology*, *116*(4), 1271–1278.

Yamauchi, Y., Ogawa, M., Kuwahara, A., Hanada, A., Kamiya, Y., & Yamaguchi, S. (2004). Activation of Gibberellin Biosynthesis and Response Pathways by Low Temperature during Imbibition of *Arabidopsis thaliana* Seeds. *The Plant Cell*, *16*(2), 367–378.

Zanoli, P., Rivasi, M., Zavatti, M., Brusiani, F., & Baraldi, M. (2005). New insight in the neuropharmacological activity of *Humulus lupulus* L. *Journal of Ethnopharmacology*, *102*(1), 102–106.

Zhai, N., & Xu, L. (2021). Pluripotency acquisition in the middle cell layer of callus is required for organ regeneration. *Nature Plants*, *7*(11), 1453–1460.

Zhang, K., Novak, O., Wei, Z., Gou, M., Zhang, X., Yu, Y., Yang, H., Cai, Y., Strnad, M., & Liu, C.-J. (2014). *Arabidopsis* ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. *Nature Communications*, *5*(1), 3274.

Zhang, N., & Laux, T. (2018). Epigenetically jump starting de novo shoot regeneration. *The EMBO Journal*, *37*(20), e100596.

Zhang, T.-Q., Lian, H., Tang, H., Dolezal, K., Zhou, C.-M., Yu, S., Chen, J.-H., Chen, Q., Liu, H., Ljung, K., & Wang, J.-W. (2015). An Intrinsic MicroRNA Timer Regulates Progressive Decline in Shoot Regenerative Capacity in Plants. *The Plant Cell*, *27*(2), 349–360.

Zhang, T.-Q., Lian, H., Zhou, C.-M., Xu, L., Jiao, Y., & Wang, J.-W. (2017). A two-step model for de novo activation of *WUSCHEL* during plant shoot regeneration. *The Plant Cell*, *29*(5), 1073–1087.

Zheng, Y., Ren, N., Wang, H., Stromberg, A. J., & Perry, S. E. (2009). Global Identification of Targets of the *Arabidopsis* MADS Domain Protein AGAMOUS-Like15. *The Plant Cell*, *21*(9), 2563–2577.

Zhong, G.-Y., & Yang, Y. (2012). Characterization of grape Gibberellin Insensitive1 mutant alleles in transgenic *Arabidopsis*. *Transgenic Research*, *21*(4), 725–741.

Zhu, Y., Nomura, T., Xu, Y., Zhang, Y., Peng, Y., Mao, B., Hanada, A., Zhou, H., Wang, R., Li, P., Zhu, X., Mander, L. N., Kamiya, Y., Yamaguchi, S., & He, Z. (2006). *ELONGATED UPPERMOST INTERNODE* Encodes a Cytochrome P450

Monooxygenase That Epoxidizes Gibberellins in a Novel Deactivation Reaction in Rice. *The Plant Cell*, *18*(2), 442–456.

Zierau, O., Gester, S., Schwab, P., Metz, P., Kolba, S., Wulf, M., & Vollmer, G. (2002). Estrogenic activity of the phytoestrogens naringenin, 6-(1, 1-dimethylallyl) naringenin and 8-prenylnaringenin. *Planta Medica*, *68*(05), 449–451.

Ziv, M. (1991). Vitrification: Morphological and physiological disorders of in vitro plants. In P. C. Debergh & R. H. Zimmerman (Eds.), *Micropropagation: Technology and Application* (pp. 45–69). Springer Netherlands.

Zuo, J., Niu, Q.-W., Frugis, G., & Chua, N.-H. (2002). The *WUSCHEL* gene promotes vegetative-to-embryonic transition in *Arabidopsis*. *The Plant Journal*, *30*(3), 349–359.

Appendix A. Supplementary tables

Table A.1. Regulators of in vitro shoot regeneration and somatic embryogenesis in Arabidopsis. General abbreviations include: cytokinin (CK); lateral root meristem (LRM); loss-of-function (LOF); overexpression (OE); shoot apical meristem (SAM), Somatic embryogenesis (SE); shoot induction media (SIM), transcription factor (TF).

Abbreviated Name	Accession No.	Gene Product	Notes	References
AGL15	AT5G13790	MADS box	Embryo expressed; SE	(Boutilier et al., 2002)
AHK4/WOL	AT2G01839	Histidine kinase receptor	Direct CK receptor; positively regulates CK signaling during shoot formation	(Higuchi et al., 2004; Su et al., 2015)
ALF4	AT5G11030	Cytokinin signaling	Required for the first asymmetric division of xylem pole pericycle cells during LR initiation; LOF blocks callus formation	(Celenza et al., 1995; Sugimoto et al., 2010)
ARF3	AT2G33860	Auxin response TF	Indirectly inhibits CK biosynthesis; mutations cause disruptions in organ regeneration	(Cheng et al., 2013)
ARR1	AT3G16857	Type-B *Arabidopsis* response regulator	Transcriptional activator of cytokinin signaling pathway; spatially activate WUS; can regenerate shoots in the absence of CK	(Sakai et al., 2001; Zhang et al., 2017)
ARR10,12	AT4G31920, AT2G25180	Type-B *Arabidopsis* response regulator	Transcriptional activator of cytokinin signaling pathway; spatially activate WUS; LOF results in impaired shoot regeneration	(Zhang et al., 2015, 2017)
ARR7,15	AT1G19050, At1G74890	Type-A *Arabidopsis* response regulator	Repressor of CK signaling; OE results in suppression of shoot regeneration	(Boutilier et al., 2002; Buechel et al., 2010; Leibfried et al., 2005)
BBM/PLT4	AT5G17430	AP2/ERF	Embryo and LRM expressed; SE	(Horstman, Li, et al., 2017; Jha & Kumar, 2018; Karim et al., 2018)
CLV3	AT2G27250	CLAVATA3/ESR-related (CLE) domain	Meristem maintenance; WUS-induced secreted peptide in SAM	(Brand et al., 2000; Schoof et al., 2000; Yadav et al., 2011)
CLV1,2		LRR kinase receptor	SAM maintenance; CLV1 and CLV2 forms a receptor complex that limits WUS activity in SAM	(Brand et al., 2000; Schoof et al., 2000; Yadav et al., 2011)
E2Fa	AT2G36010	E2F; TF	Positive regulator of the cell cycle; callus and SAM expressed; OE inhibits shoot regeneration	(H. Liu et al., 2018)
ESR1/DRN	AT1G12980	AP2/ERF	LRM-SAM transition; *ESR1* expressing cells proliferate to form	(Banno et al., 2001; Matsuo et al., 2011)

			SAM; OE enhances shoot regeneration	
ESR2/DRNL	AT1G24590	AP2/ERF	LRM-SAM transition; LOF severely effects shoot regeneration	(Banno et al., 2001; Matsuo et al., 2011)
GRF5	AT3G13960	Growth Regulating Factor	Forms transcriptional complex with its cofactor *GRF-INTERACTING FACTOR* to provide cues in primordial cells; ectopic expression enhances regeneration and recovery of transgenic plants	(Kong et al., 2020; Luo & Palmgren, 2020)
HAG1	AT3G54610	Histone acetyltransferase	Conveys competency for shoot regeneration in callus tissue by promoting the expression of root stem cell factors	(Kim et al., 2018; Zhang & Laux, 2018)
IAA30	AT3G62100	AUX/IAA family	Auxin induced; accumulates in the quiescent center of the root meristem; target of *LEC2* and *AUG15*; promotes SE	(Zheng et al., 2009)
IPT3,5	AT3G63110, AT5G19040	Cytokinin synthase	Negative regulation determines spatial auxin-cytokinin cross-talk; pro-meristem formation; mutations reduce shoot regeneration efficiency	(Cheng et al., 2013)
LBD16	AT2G42430	LATERAL ORGAN BOUNDARIES DOMAIN; TF	Root meristem regulator; callus formation; acquisition of competency for shoot regeneration	(Fan et al., 2012)
LEC1	AT1G21970	Subunit B of nuclear factor Y protein	Cotyledon identity and activator of embryo development; *BBM*-induced; SE	(Horstman et al., 2017; Lotan et al., 1998)
LEC2	AT1G28300	B3 domain TF	Cotyledon identity and completion of embryo maturation; *BBM*-induced; SE	(Horstman, Li, et al., 2017; Stone et al., 2001)
MET1	AT1G55480	DNA methyltransferase	Represses shoot regeneration via *WUS* inhibition	(Liu et al., 2018)
MNP (ARF5)	AT1G19850	Auxin response TF	Embryo axis formation and vascular differentiation; *MP* variant that lacks inhibitory domains promotes *de novo* shoot organogenesis	(Ckurshumova et al., 2014; Przemeck et al., 1996)
MiR160	AT1G77850	MicroRNA	Down-regulated on CK-rich SIM; OE inhibited shoot regeneration	(Qiao et al., 2012)
PHB	AT2G34710	HD ZIP III	Cytokinin induced LRM-SAM transition; *phb,phv* mutant has compromised shoot regeneration	(Zhang et al., 2017)
PHV	AT1G30490	HD ZIP III	Cytokinin induced LRM-SAM transition; *phb,phv* mutant has compromised shoot regeneration	(Zhang et al., 2017)
PKL	AT2G25170	CHD3 chromatin remodelling group	Represses embryo development post-germination; inhibits *LEC1* during vegetative development	(Ogas et al., 1999)
PLT1,2	AT3G20840, AT1G51190	AP2/ERF	Root stem cell regulator; convey competency for shoot regeneration in callus tissue	(Kareem et al., 2015)

PLT3,5,7	AT5G10510, AT5G57390, AT5G65510	AP2/ERF	Induce *PLT1, PLT2, CUC1, CUC2* expression; convey competency for shoot regeneration in callus tissue	(Kareem et al., 2015)
PRMT5	AT4G31120	Arginine methyltransferase	Histone modification and pre-mRNA splicing; shoot regeneration and shoots per callus were reduced in LOF mutant	(Liu et al., 2016)
RAP2.6L	AT5G13330	AP2/ERF	Transcript accumulation at shoot initiation sites; LOF results in reduced regeneration capacity	(Che et al., 2006)
REV	AT5G60690	HD ZIP III	Cytokinin induced LRM-SAM transition; shoot regeneration is abolished in the *phb,phv,rev* mutant	(Zhang et al., 2017)
SCR	AT3G54220	GRAS	Root stem cell regulator; convey competency for shoot regeneration in callus tissue	(Kareem et al., 2015; N. Zhang & Laux, 2018)
SERK1,2	AT1G71830, AT1G34210	Transmembrane LRR receptor kinase	Embryo expressed; early acquisition of embryogenic competence during SE	(Hecht et al., 2001; Karim et al., 2018)
STM	AT1G62360	KNOX	Required for SAM initiation; inhibits the incorporation of meristem cells into developing organs	(Aida et al., 1999; Gordon et al., 2007; Zhang et al., 2017)
WIND1	AT1G78080	AP2/ERF	Central role in callus formation; promotes shoot regeneration through the activation of *ESR1*; SE	(Iwase et al., 2017)
WOX5	AT3G11260	Homeobox	Root meristem regulator; *WUS* paralog; acquisition of competency for shoot regeneration	(Zhai & Xu, 2021)
WOX7,11,12,14	AT5G05770, AT3G03660, AT5G17810, AT1G20700	Homeobox	Root meristem regulator; acquisition of competency for shoot regeneration	(Liu et al., 2014)
WUS	AT2G17950	Homeobox	Establish and maintain stem cell populations in SAM; vegetative-embryonic transition; GOF improves shoot regeneration	(Cheng et al., 2013; Gordon et al., 2007; T.-Q. Zhang et al., 2017; Zuo et al., 2002)
YUC1,4	AT4G32540, AT5G11320	Auxin biosynthesis	Yucca-mediated auxin biosynthesis is required for shoot regeneration; promotes SE	(L. Chen et al., 2016; Wójcikowska et al., 2013)

Table A.2. Summary of plant culture media. All media contained a base of 1x MS with 0.1% MES, 2% Glucose, 0.6% plant agar; buffered to pH 5.8. Exception: media Co.L was absent of plant agar.

Media	Plant hormones	Additives	Purpose
PPM	-	-	Plant propagation
R1a	9 μM TDZ, 1.4 μM IAA	-	Shoot regeneration
R1b	4.6 μM Zea, 1.4 μM IAA	-	Shoot regeneration
R1c	4.6 μM Zea	-	Shoot regeneration
R1d	8.8 μM BAP	-	Shoot regeneration
R2a	5 μM TDZ, 1.4 μM IAA	-	Shoot multiplication
R2b	5 μM Zea, 1.4 μM IAA	-	Shoot multiplication
R2c	5 μM BAP, 1.4 μM IAA	-	Shoot multiplication
R3	0.5 μM IBA	-	Rooting
Co.L	none	200 μM Acetosyringone	Liquid co-culture
Co.A	9 μM TDZ, 1.4 μM IAA	200 μM Acetosyringone	Solid co-culture

Table A.3. Summary of primers used in the study. Nucleotides highlighted in red complement the cloning site for ligation. Primer pairs for housekeeping genes *7sl-RNA* and *GAPDH* were obtained from Maloukh et al., 2009.

Primer Name	Sequence (5'-3')	Orientation	Function
Hpt_F	ATTTCATATGCGCGATTGCT	Forward	Genetic screening
Hpt_R	GATGTTGGCGACCTCGTATT	Reverse	Genetic screening
BBM_F	CCCTCCGATGAATCTTCTGC	Forward	Genetic screening
BBM_R	GTCCCATAAATGTGCCTCGT	Reverse	Genetic screening
WUS_F	AACAGCCGATCAGATCCAGA	Forward	Genetic screening
WUS_R	AGAAGCGCAAGGGCGAA	Reverse	Genetic screening
Cas9_F	CTTTCCTGTCTGGGGAGCAG	Forward	Genetic screening
Cas9_R	TCGACGTCGTAATCGCTGAG	Reverse	Genetic screening
GA20ox1_F	TGACGATCATCGGAAACAGC	Forward	Sequencing
GA20ox1_R	CGCTTTGCTATTCACCACCG	Reverse	Sequencing
GA20ox2_F	TGCGTGTTTACTGTGCGTTT	Forward	Sequencing
GA20ox2_R	CTGGGTAAAGGCATTGAGGGT	Reverse	Sequencing
qGA20ox1_F	ACCGAGCGGTGGTGAATAGCA	Forward	qRT-PCR
qGA20ox1_R	ACCCGAGGGCTTGAATGGTCC	Reverse	qRT-PCR
qGA20ox2_F	GGGGATGAGCCTAGGAGTTGGG	Forward	qRT-PCR
qGA20ox2_R	AGTGAGGCCCAGTCCCCAGTG	Reverse	qRT-PCR
qGA20ox3_F	ACCTGGCGCAAAAGCACATGG	Forward	qRT-PCR
qGA20ox3_R	TGGACGACCGGAACAGAGCA	Reverse	qRT-PCR
qGA20ox4_F	ACGGGGCAGCCTTTCAGGTT	Forward	qRT-PCR
qGA20ox4_R	GGTAGACCCACCCAGCGTGT	Reverse	qRT-PCR
qGA20ox5_F	AGCCTCGGAGTAGGCAGAGC	Forward	qRT-PCR
qGA20ox5_R	TCCTCCCACTTGGTGTTGGTGG	Reverse	qRT-PCR
qGA3ox1_F	GCCACAAAACCTCTCATGGCCC	Forward	qRT-PCR
qGA3ox1_R	GGAGTCCGTGTGAGCAGCCA	Reverse	qRT-PCR
qGA3ox2_F	ACCACGGCATCCCAACCCAC	Forward	qRT-PCR
qGA3ox2_R	GACGCCATCAGGGGAGCGAG	Reverse	qRT-PCR
qGA3ox3_F	TTGATGTTCGGGTCACTCGGCT	Forward	qRT-PCR
qGA3ox3_R	CGTGTGAGCAGCCAAGCCAAT	Reverse	qRT-PCR
7sl-RNA_F	TGTAACCCAAGTGGGGG	Forward	qRT-PCR
7sl_RNA_R	GCACCGGCCCGTTATCC	Reverse	qRT-PCR
GAPDH_F	ACCGGAGCCGACTTTGTTGTTGAA	Forward	qRT-PCR
GAPDH_R	TCGTACTCTGGCTTGTATTCCTTC	Reverse	qRT-PCR
GA20ox1.sp1_F	**ATTG**GGAAAGTAGGGGAACACTG	Forward	gRNA spacer cloning
GA20ox1.sp1_R	**AAAC**CAGTGTTCCCCTACTTTCC	Reverse	gRNA spacer cloning
GA20ox1.sp2_F	**ATTG**GGCTTCTTGTCCGGTGACC	Forward	gRNA spacer cloning
GA20ox1.sp2_R	**AAAC**GGTCACCGGACAAGAAGCC	Reverse	gRNA spacer cloning
GA20ox2.sp1_F	**ATTG**CAGATCTAACACTGGGGAC	Forward	gRNA spacer cloning
GA20ox2.sp1_R	**AAAC**GTCCCCAGTGTTAGATCTG	Reverse	gRNA spacer cloning
GA20ox2.sp2_F	**ATTG**AGGATCTCACTTGGGGTGA	Forward	gRNA spacer cloning
GA20ox2.sp2_R	**AAAC**TCACCCCAAGTGAGATCCT	Reverse	gRNA spacer cloning
U6_F	TGTCCCAGGATTAGAATGATTAGGC	Forward	gRNA cloning confirmation
U6_R	GTTCTTCGGCGTTCAATTTCTGGGG	Reverse	gRNA cloning confirmation
SLIC_F	**gattgacaacgaatt**TGATATCTCCACTGACGTAAGGG	Forward	SLIC cloning
SLIC_R	**acatgattacgaatt**ACGACGGCCAGTGAATTCC	Reverse	SLIC cloning
SLICseq_F	GTGGCCTCTAATGACCGAAG	Forward	SLIC cloning confirmation
M13_R	AGCGGATAACAATTTCACACAGG	Reverse	SLIC cloning confirmation
VirE3_F	CGTCAAACCTCCCAGACGAA	Forward	EHA105 confirmation
VirE3_R	AGATTTCCACTCGGGTGTGC	Reverse	EHA105 confirmation
VirD5_F	CTGAAACGAGGCCTGTGCAA	Forward	EHA105 confirmation
VirD5_R	CCGAGCGATAGCTTGTGTCA	Reverse	EHA105 confirmation

Appendix B. Supplementary figures

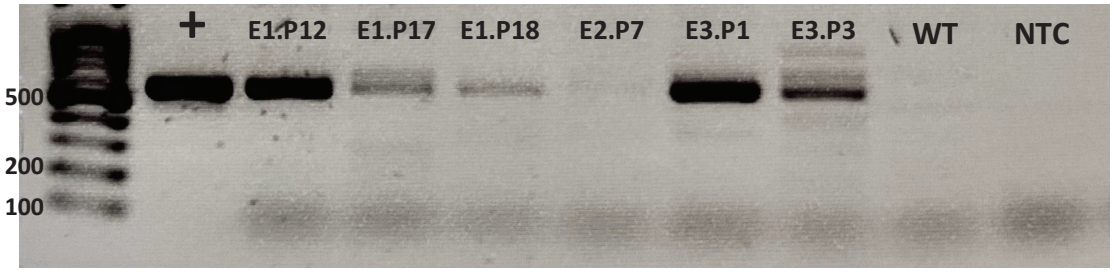

Figure B.1. PCR genotyping of AtBBM shoots. Secondary screening (>1 year) of shoots obtained from *p35SBBM* transformations with *AtBBM*-specific primers. Expected product = 552 bp. The *p35SBBM* plasmid was used as a positive control and wildtype (WT) DNA served as a negative control. Our no template control (NTC) consisted of water substitution for DNA template.

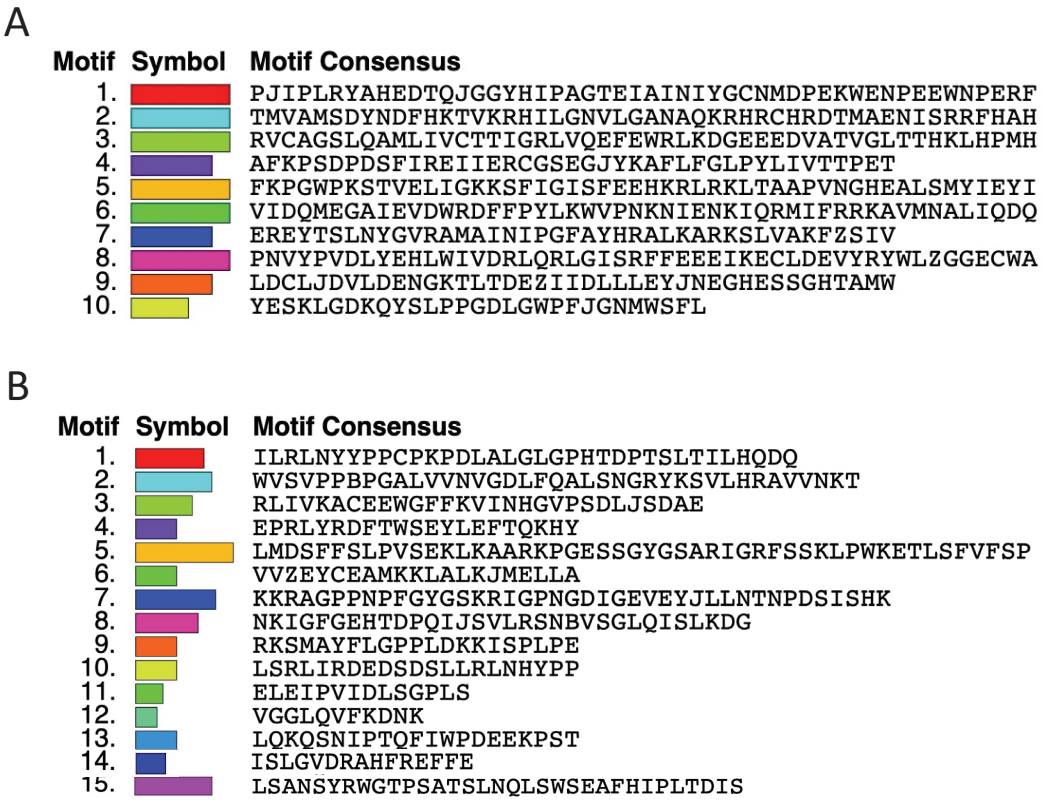

Figure B.2. Conserved motifs. Early GA biosynthesis enzymes (A) and GAox enzymes (B). Ungapped motifs were identified in the online software MEME with the following parameters: max motif count = 15; motif width 6-50 amino acids.

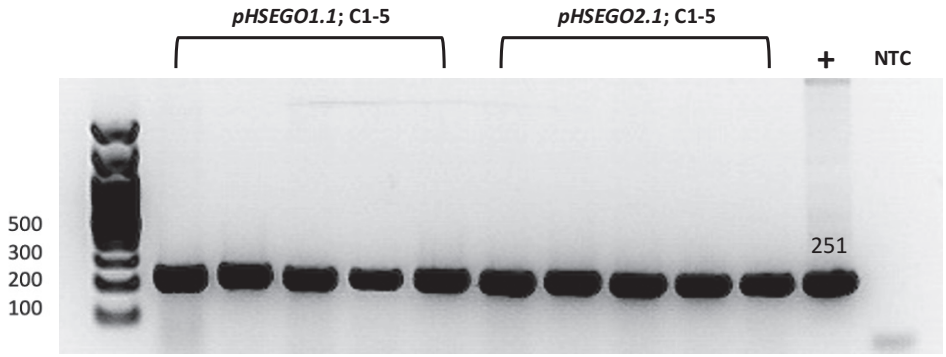

Figure B.3. PCR screening of pHSE401 transgenic callus. Callus (C) tissue was selected from *pHSEGO1.1* and *pHSEGO2.1* transformations and PCR screened for the presence of the *Hpt* gene after 6 months of sub-culturing on R1a with 200 mg/L Timentin. Expected product: 251 base pairs. Samples are listed 1-5 for each vector. Positive control included the *pHSEGO2.1* vector and the negative control consisted of a no template control (NTC).