EXPLORING THE MECHANISMS CONTROLLING EMBRYOGENIC TISSUE CULTURE RESPONSE AND REGENERATION ABILITY IN MAIZE

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OVERALL ABSTRACT

The ability to form embryogenic cultures and regenerate green plants *in vitro* is a critical factor in plant genetic engineering, plant propagation and transformation-based research. Maize regeneration ability is genotype-dependent and limiting to tissue culture-based methodologies. The overall goal of this research is to further our understanding of tissue culture response in maize. A backcross-derived mapping population between the highly embryogenic and regenerable maize genotype, A188 was crossed to the poorly culturable maize reference inbred line B73. Near-isogenic lines were screened for tissue culture response. High-resolution mapping in this material to further refine the position of genes on chromosome 3 conferring culture response resulted in two flanking single nucleotide polymorphic markers with a physical distance between markers of 3,053kb or a genetic distance of 4 centimorgans, based on recombination frequency in segregating F_2 individuals (n=2243). A fine-mapping experiment revealed highly significant marker-trait associations ($P<0.0001$) for multiple tissue culture traits measured in homozygous recombinant plants (n=128) on F_4 embryos (n=6400) such as callus diameter, number of zygotic embryos displaying somatic embryogenesis, and number of plantlets regenerated in tissue culture. In addition to genetic mapping, research was also undertaken to examine the transcriptional profile in the early stages of culture initiation from immature embryos of the highly embryogenic and regenerable maize genotype A188. Gene expression levels in immature embryos collected at different time points (0, 24, 36, 48 and 72 hours after plating in culture) were analyzed via RNA-Seq analysis. Several somatic-embryogenesis related genes were altered in expression. This transcriptome analysis provides information on genes expressed during early embryogenesis that may play a role in somatic embryo formation in

maize and other plant species, and is providing information that is complementary to our genetic mapping studies.

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CHAPTER 1: A REVIEW OF THE LITERATURE

As the world faces an era of unprecedented climate change, malnutrition, and population growth, a major role for scientists, policy makers, and global leaders will address how to feed nine billion [1-3]. The polarizing debate on genetically modified organisms continue to be complex [4]. Academic researchers will aid the discussion by providing knowledge-based solutions that guide policy and public education [5,6]. The Food and Agriculture Organization of the United Nations have spearheaded a new initiative called climate-smart agriculture [7]. This concept is an attempt to unify the objectives in food production that incorporates local and global economics, social concerns and capabilities, and environmental outcomes and strategies. The climate-smart agriculture initiative includes biotechnology. Basic and applied research on biotechnology-related advances, such as the work presented in this dissertation, continue to be an important part of the future of food [8].

Important applications of somatic embryogenesis and plant regeneration ability in tissue culture

Gaining an understanding of somatic embryogenesis and plant regeneration ability has positive implications for crop improvement, functional genomics research, plant propagation and other potential future approaches towards improving breeding processes and implementing efficiencies in agriculture. Genetic engineering has benefited from the study of improved regeneration ability and has an important role in agriculture [8]. The ability to conduct transformation-based research in functional genomics has allowed for a targeted approach to candidate gene testing and introgression by genetic modification. Successful incorporation of regeneration ability genes into otherwise recalcitrant plant species has been reported in sweet pepper and Chinese white poplar [9,10]. Clonal vegetative propagation is widely used in

ornamental crops and trees species as a method for maintaining disease free stocks *in vitro* [11], increase production en masse in a relatively short period of time, and improve efficient material transfer with again, large quantities, and with relative ease [12].

Potential future uses for plant tissue culture that would benefit from an increased understanding of early somatic embryogenesis and the necessary genetic and physiological components of regeneration ability include the possibility for synthetic seed technology. Benefits of synthetic seed technology are similar to those of micropropagation such as the ability to efficiently produce a large number of identical clones *in vitro*. Moreover, since callus cultures often have many embryos at different stages of embryogenic development, this asynchronous nature of callus maintenance would allow for a constant production supply of somatic embryos and clones [13]. Other applications for synthetic seed technology could be to overcome long breeding cycles, decrease the cost of seed production by mass producing hybrids, *in situ* conservation of plant germplasm or germplasm exchange, and in some cases overcoming self-incompatibility, reducing the need for inbred lines. Somatic embryos could be encapsulated to ensure protection during transport [13] for applications *in vitro* and in the field. A limitation to the widespread use of synthetic seeds is the potential for somaclonal variation [14] in long-term callus tissue cultures. In addition, the initial startup costs of automating cell culture and encapsulation may be very high depending on the plant species [13], and the widespread use of synthetic seeds in the field that requires added treatment to trigger germination may be costly and difficult to establish [13].

In addition, another future application of somatic embryogenesis and plant tissue culture is in rapid cycle breeding that enables a "cycling of gametes *in vitro"* (COGIV). QTL analysis, marker-assisted selection, and genomic selection could be incorporated with COGIV to expedite

the breeding process [15]. Currently, the pace of agricultural productivity is inadequate for the future of food, feed and fiber production [2]. Some of the benefits of the proposed COGIV approach are: the ability to bypass sporophytic incompatabilities, broadening genetic variation, enabling multiple generations a year, enabling precision phenotyping and automation and most importantly, reducing generation time [15]. A current limitation to this approach is the necessary induction of somatic cells into gametes. Improving our understanding of somatic embryogenesis and the ability of somatic cells to dedifferentiate into different organs would enable COGIV to become realized. In addition, overcoming genotype specificity would also enable COGIV.

The early tissue cultures of maize

The first documented plant tissue culture was initiated in 1934 from lateral vascular meristematic tissue of a maple tree [11]. Since the first tissue culture studies to the present, tissue culture research has been involved in the study of plant morphology, physiology, biochemistry and molecular genetics. Early tissue culture research in maize utilized non-regenerable callus cultures initiated from stem sections of 6 day old seedlings of the maize genotype Black Mexican Sweet (BMS) [16]. Because of its relatively fast growth rate and friable callus morphology, BMS callus cultures were commonly used and propagated as fine cells in suspension culture. BMS-based studies included investigations on plant cell tissue culture optimization [17], research on cell cycle time and cytology [18], and transformation with protoplasts [19] by electroporation [20,21] that was used to optimize and study important components in vector construction [22]. By the late 1980's to early 1990's, after the discovery of a maize inbred line A188 [23,24] capable of producing highly embryogenic, regenerable callus cultures, studies evolved to focus on embryogenesis, plant regeneration and transformation to obtain fertile plants, however other methods to promote transformation still included cell suspension culture systems such as

transformation with silicon carbide fibers [25,26] and microprojectile bombardment [27], and transformation with selectable markers such as bialaphos [28]. Finally, transformation with *Agrobacterium tumefaciens* leading to the introduction of foreign genes into plant cells using kanamycin-resistance, for example, paired with the ability and focus on regeneration ability to produce fertile transgenic plants, led to the commercial production of genetically modified crop plants [29]. These studies were crucial to the forward progression of genetic engineering and plant biotechnology. Today, among other applications, tissue culture is a model system to study the onset and initiation of embryo development in plants [30].

Somatic embryogenesis

The most commonly used tissue culture explants in maize are immature zygotic embryos derived from derived from responsive genotypes that are capable of somatic embryogenesis [22]. The process of embryogenic callus initiation involves characteristic morphological and biochemical events that allow the plant cells to dedifferentiate or transition into whole plants [31]. Little is known about specific pathways leading to somatic embryogenesis, but the morphological phases of embryo development have been described. Zygotic embryo development in dicots such as Arabidopsis can be described in stages by what the embryo may look like during development after fertilization: globular-shaped, heart-shaped, torpedo-shaped, cotyledonal [31-34]. In monocots such as maize, embryogenic growth can be described in two main phases [35]. The first phase begins with the transition marked by the embryo proper and suspensor. The second phase begins with the developing coleoptilar stage marked by the visible scutellum, coleoptile, shoot apex and suspensor. Subsequent stages in maize embryo development lead to the appearance of leaf primordia, root primordia, coleorhiza and mesocotyl [35].

In Arabidopsis, distinct phases in zygotic embryogenesis are described by three major events [32,36,37]. The initial asymmetric cell division that gives rise to the apical cell that becomes the embryo and the basal cell that becomes the suspensor. This apical-basal orientation is then followed by pattern formation or the differentiation of meristematic tissue such as the shoot apical meristem, or the shoot apex, and the organizing body of cells known as the organizing center. Meristematic tissue is important for establishing the identity and maintenance of embryogenic stems cells in embryo development and in tissue culture [36-38]. Finally, the transition to the cotyledon state and the initiation of root and shoot primorida gives rise to mature embryo ready for germination. It has been suggested that the first stages up to the globular stage in dicots and up to the transition phase in monocots share similarities in regulatory mechanisms involved in both zygotic and somatic embryogenesis [32,39]. In general, those similarities in the onset of embryogenesis and acquiring embryogenic competence involve responsive cells. These cells have the potential and the totipotency to activate genes involved in reprogramming somatic cells leading to cellular division and proliferation [31,39,40].

Plant tissue culture media

Plant tissue culture media are composed of essential elements such as macronutrients (eg. N, P, K, Ca, etc.), micronutrients (eg. Cu, Zn, Mn, etc.), carbohydrates, hormones, and vitamins. For maize tissue culture, two main formulations of macronutrient and micronutrient basal salts have been used extensively, the Murashigie and Skoog (MS) and Chu's N6 basal salt formulations. MS-based tissue culture mediun was first developed for tobacco cultures [41], and Chu's N6-based mediun was originally developed for rice anther cultures [42]. Some of the key differences between the two basal salt mixtures are an increased amount of ammonium nitrate, potassium nitrate and potassium phosphate in MS salts compared to N6 salts, respectively.

Finally, other crucial components of plant tissue culture media are sugars or sucrose, a fixed carbon necessary for cellular growth and development, and water [43]. Plant growth regulators or synthetic plant hormones are also important for successful tissue culture response. These plant growth hormones include auxin to promote cell division and cell growth, cytokinins to promote cell division, gibberellins to regulate cell elongation, and abscisic acid to inhibit cell division that promotes somatic embryogenesis [43]. Tissue culture media formulations for maize and other plant species have been thoroughly studied and improved to include optimal amounts of other components. For example, the addition of L-proline to N6 medium increases callus initiation and somatic embryo formation in maize possibly by playing a role in improving plant metabolism as an added source of nitrogen or improving solubility in tissue culture [24]. Exogeneous auxin in the form of 2,4-dichlorophenoxyacetic acid (2, 4-D) effectively stimulates callus induction and somatic embryo formation [44], and the addition of silver nitrate in tissue culture medium aids in reducing ethylene in the culture environment [45] also improving embryogenic callus formation in maize.

Genotype specificity in maize

Maize is a model species for plant breeding and plant genetics research [46] and also an economically important crop worldwide. The ability to efficiently regenerate fertile plants from embryogenic cell cultures is a critical requirement of current genetic engineering-based research such that studies focusing on screening for maize germplasm with the capacity to regenerate plants *in vitro* remains an important objective [47-54]. Previous maize tissue culture studies showed that very few maize genotypes are efficient in embryogenic callus formation and plant regeneration [55-57]. It is still not widely understood why only specific genotypes in maize and

in other plant species, such as peanuts and soybeans, are capable of efficiently growing in vitro while other genotypes are not [57,58].

Important factors that influence establishing successful and efficient embryogenic, regenerable tissue culture response using immature zygotic embryos as the explant source are the genotype and overall health of the zygotic embryo donor plant, the size and stage of development of the immature embryo for initiating cell culture, the media formulations, and the orientation of the zygotic embryo upon placement on the tissue culture medium [23,30,59]. In maize, embryogenic callus formation and morphology are distinguishable and are typically described as either Type I or Type II calli. The more preferred calli type are Type II calli, which are light yellow to white in color, grow relatively fast, and are highly friable and embryogenic and easy to subculture [60]. Type I calli are typically less efficient in regeneration ability. The calli are usually yellow, hard, compact, slow growing, form few or no somatic embryos (which, if present, are often fused) and are more prone to tissue culture induced variation or somaclonal variation [60]. Tissue culture-induced variation or somaclonal variation caused by stress induced changes in cell division during cellular growth and maintenance are not optimal for stable transformation and fertile plant regeneration [61,62]. Somaclonal variation causes a decline in callus culture health over time and is suggested to be caused by genetic or epigenetic changes over time that can also cause phenotypic variation in clonally propagated plants [63]. These changes are induced by the tissue culture growing environment. When cells are grown *in vitro*, cellular division must undergo dedifferentiation which involves massive stress-induced genomic program changes leading to adaptation. Some of these adaptive changes are due to genetics and epigenetics.

Successful plant regeneration in tissue culture for maize inbred line, A188, was first described in 1975 [23]. A188 has exemplary Type II culture response and regeneration ability. The inbred has been utilized in many studies aimed at understanding and improving embryogenic efficiency and regeneration ability in maize tissue culture. For example, A188 was used to study improvements to tissue culture response by crossing A188 to other maize lines representing different heterotic groups in maize [55]. A188 was used to assess allele frequency changes in a population studied to a determine the feasibility of using a recurrent selection breeding strategy to improve tissue cultureability [64,65]. A188 was also used to demonstrate improved tissue culture response through marker-assisted backcross breeding [66]. These studies have suggested that the genes associated with embryogenic, regenerable tissue culture response act in an additive manner and that one or a few major genes may be involved in regeneration ability in maize.

Biological functions and candidate genes involved in somatic embryogenesis and regeneration ability

To date, there are no known causal genes for embryogenic, regenerable tissue culture response identified and characterized in maize. In addition, there is no specific pathways identified that has proven to be integral to the induction of somatic embryogenesis and regeneration ability in maize and in other plant species. There are some important biological functions that take place during the tissue culture process that have been widely accepted [39,40,67,68]. Stress response in tissue culture acts as a genomic trigger in the initiation of somatic embryogenesis and is suggested to be an important part of the transition of somatic cells to embryogenic cells [40]. Other biological functions that may be related to tissue culture

response include the disruption of cell cycle regulation, factors influencing plant growth regulation and metabolism, or chromatin remodeling and epigenetic modifications [40,69-72].

In Arabidopsis, transcription factors that have been studied during embryogenesis and are potential candidate genes that promote somatic embryogenesis are BABY BOOM (BBM) [73] and LEAFY COTYLEDON2 (LEC2) [74]. BBM was identified in microspore cultures by subtractive hybridization. Overexpression of BBM in transgenic plants led to spontaneous formation of somatic embryos in Arabidopsis [73], Chinese white poplar [9], and sweet pepper [10]. LEC2 when overexpressed in transgenic Arabidopsis plants was also shown to promote somatic embryo formation [74,75].

Other genes identified in Arabidopsis originally as homeotic genes have also been associated with embryogenesis in the maintenance and initiation of stem cells regulating pathways that control meristematic tissue and cell division. Some of these genes are AGAMOUS, a MADS-box transcription factor [76], CLAVATA a receptor kinase [77] that acts in a regulatory loop with WUSCHEL, a homeodomain protein which acts to regulate and establish a stem cell niche that leads to somatic embryo formation and callus maintenance [78-80]. Plant growth regulators or hormone-response genes such as PINFORMED1, a PIN gene involved in polar auxin transport in normal seed development has been suggested to also be important for auxin mediated functions in somatic embryogenesis [81-83].

Currently, there are no genes reported in maize that are directly responsible for somatic embryogenesis and regeneration ability. Few studies have been done to identify and characterize Arabidopsis orthologs involved in embryogenesis in maize tissue culture. *ZmSERK*, homologous to SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK) originally described in

Arabiodopsis [84,85] was characterized during callus formation [86]. ZmSERK was found to increase in expression when enhanced with additional auxin [87]. Although SERK genes have successfully marked embryogenic competence in other plant species such as carrot and Arabidopsis, this is not the case for maize. In maize, SERK gene expression is detected in both embryogenic and non-embryogenic callus.

In an attempt to understand important signaling pathways involved in the onset of somatic embryogenesis and regeneration ability, one approach altered the pathway regulating cellular processes *in vitro* via transformation. A replication-associated protein (RepA) involved in cell cycle regulation was introgressed into maize by both particle bombardment and *Agrobacterium*-mediated transformation [88]. Altering normal cell cycle regulation gave rise to an increase in callus growth rate and improved transformation frequency.

Comprehensive studies on candidate genes and expression networks

Comprehensive studies on candidate genes and expression networks has improved the understanding of somatic embryogenesis in maize and in other plants. A study in cotton, also limited by genotype specificity in embryogenesis, used bioinformatics to draft a regulatory network of cellular reprogramming associated with totipotency and somatic embryo development [89]. Somatic embryogenesis-related genes were identified by subtractive hybridization [12] and then used as major nodes in a meta co-expression network. This study leveraged open access public databases to gain an understanding of the complexity of the many different pathways and biological functions that were found to be enriched in this data set. Whole genome transcriptome studies on somatic embryogenesis of maize in tissue culture have also shed light on the various stages of embryogenesis from initiation to embryogenic callus. One study focused on the HiII

hybrid. HiII was created by germplasm development using the highly cultureable maize inbred line A188 and B73 [90] and, HiII is widely used for maize tissue culture today. Differences in gene expression of HiII was analyzed using a microarray-based platform to study embryogenic callus at various stages of development [91]. This study identified genes with significant variation in expression at different stages of sampling from 7 to 28 days after plating on tissue culture. This study also discussed gene expression trends of some of the major biological functions associated with embryogenesis. Another study used next generation sequencing technology to study the whole genome transcriptome profiling of a highly regenerable maize line from China, 18-599R, on samples representing three major phases after inoculation or plating on tissue culture media [92]. The first stage sampled enlarged embryos between 1-5 days after inoculation; the second stage represented samples with initial callus formation between 6-10 days after inoculation, and the final stage was a sampling of embryogenic callus 11-15 days after inoculation. This study focused on pathway enrichment of differentially expressed genes at these various stages of development. Another recent study used amplified fragment length polymorphisms (AFLP) to generate differential gene expression between embryogenic and non-embryogenic callus from the maize inbred line H99 and identified candidate genes that were then cloned and studied at various stages of development using real time qRT-PCR [93]. In rye, the expression profile of somatic embryogenesis-related genes was studied using real time RT-PCR. Rye orthologes for SERK, LEC1, VP1 and NiR were studied in a variety of samplings and compared between rye lines with high regeneration ability, L318, to lines with low regeneration ability, L9. SERK was found to always display higher expression in L9. LEC1 was also found to be expressed higher in L9 versus L318 except at 4 weeks after tissue culture suggesting that LEC1 may play an important role in regeneration ability.

QTL studies on tissue culture response

Numerous studies have been conducted in maize, wheat, rice, barley, soybean, and other plant species such as sunflower, coffee, and cucumber, to map QTL associated with tissue culture response (Table 1). A comprehensive list of QTL studies in tissue culture show differences in tissue culture methodology such as the source of explants used ranging from anther cultures, mature seeds, leaf cuttings, protoplasts, and immature embryos. The QTL studies also differed within plant species in genotypes studied with high tissue culture response and phenotypes measured such as callus formation, number of somatic embryos and green plantlets regenerated. Finally, QTL studies in general, typically also differ in the type of markers used and the structure of the mapping population used to conduct the studies.

To date, only one QTL study was successful in identifying a gene associated with regeneration ability in tissue culture. A ferredoxin-nitrite reductase (NiR) gene in rice was cloned from a large effect QTL originally identified in a backcross-derived mapping population [94]. To confirm the effect of the cloned gene, a NiR transgene was transformed into a non-regenerable inbred line. The transgenic plants with the transgene insertion showed differential expression for regeneration ability. In this study, plants with high NiR activity were always associated with high regeneration ability [94]. The suggested mechanism for NiR activity in tissue culture was the high NiR activity in lines with efficient regeneration ability are more rapidly capable of metabolizing nitrite, which is toxic to cell culture, compared to lines with low NiR activity. Lines that did not have an increase in expression of the NiR transgene were not able to rapidly metabolize nitrite, therefore, lines lacking in NiR activity had low regeneration ability. This study showed the importance of nitrate assimilation in tissue culture response and successfully identified a gene associated with regeneration ability. However, this gene is not widely used in

improving transformation frequency and regeneration ability in rice since most rice varieties already have high NiR activity. The researchers suggest that genotype specificity in rice tissue culture is still unresolved, and that rice varieties with high NiR and poor regeneration ability have other genetic mechanisms limiting their efficiency in tissue culture [95].

CONCLUSION

The overall objective of this work was to gain a better understanding of the genetic mechanisms that confer embryogenic, regenerable tissue culture response in maize. Briefly, this dissertation focused on three main studies: (1) investigating the whole genome transcriptional profiling of genes expressed during the initial early stages of embryogenic, regenerable tissue culture response, (2) validating putative QTLs associated with embryogenic regenerable tissue culture response by testing the effect of those QTLs in near-isogenic lines, and (3) fine mapping a major QTL associated with efficient embryogenic, regenerable tissue culture response. The first goal was to analyze transcripts detected in the maize inbred line A188 during a specific developmental window when immature zygotic embryos are place on the tissue culture plating environment at 0, 24, 36, 48, and 72 H. A global analysis identified gene ontology enrichment for large fold changes and expression trends that elucidated major biological functions associated with the early stages of tissue culture response. Additionally, an in-depth review on genes identified as somatic embryogenesis-related genes was analyzed and discussed to propose a model of the major genes involved in the process. The relative gene expression trends of somatic embryogenesis related genes explained in relation to the major functions associated with early embryogenesis such as stress response, embyogenic pathway initiation, and somatic embryo formation were also highlighted. The second goal leveraged the information from previous studies on QTLs discovered in maize that were found to be associated with tissue culture

response and validated those QTLs for association with regeneration ability. In addition to QTL validation, this study used marker-assisted backcross breeding to identify near-isogenic lines harboring segments of the donor line that offer a comparison between putative QTLs. Finally, the third goal in this study was to fine map and gain higher resolution mapping to the genetic and physical location of a small segment of the donor line in a near-isogenic line that was confirmed to be capable of efficient regeneration ability. The ultimate goal of this study will be to eventually discover candidate genes that are responsible for embryogenic callus growth regeneration ability in maize that could then be used to improve plant tissue culture research and applications by overcoming the limitations due to genotype in maize and other plant species.

Table 1.1 Quantitative trait loci mapping studies aimed at identifying loci associated with tissue culture response in a variety of plant species.

Species	Trait	Populaton structure	QTL	Marker	QTL methodology	Paper
Arabidopsis	shoot formation	RIL	1, 4, 5	137 markers for genome scan, Affymetrix ATH1 oligonucleotide array	Composite interval mapping (QTL cartographer)	[96]
Barley	shoot differentiation	Backcross population, F_2	2	Isozymes	Linkage analysis	[97]
	callus growth, shoot regeneration	DH	1, 2, 3, 4	NABGMP	Interval mapping (Mapmaker/QTL)	[98]
	anther-culture response, plant regeneration rate	DH	2H, 3H, 4H	RFLP, RAPD, SSR	Segregation distortion, JoinMap	[99]
	green and albino plant regeneration	DH	1, 2, 3, 4, 5, 6, 7	North American Barley Genome Mapping Project	Mapmaker, linear regression	[100]
	callus growth, shoot differentiation, green shoot	RIL	1H, 2H, 3H, 5H, 7H	272 point markers	Mapmaker, QTL cartographer	[101]
	plant regeneration	DH	1H, 2H, 3H, 4H, 5H, 6H, 7H	Transcript-derived markers	Composite interval mapping	[102]
Brassica oleracea	protoplast regeneration	F_2	Linkage groups O2/C8, O9/C7	AFLP/modified bulked segregant analysis	Simple interval mapping (PlabQTL)	[103]
Coffee	somatic embryogensis, vegetative cutting capacity	clones, accessions, segregating progenies	Linkage group A, G	SSR	Interval mapping (MapQTL)	[104]

Maize	embryogenic embryos, plants per embryo	Backcross population, F_2	1, 2, 4, 9	RFLP	Multiple regression	[66]
	Anther culture response	DH crosses	1, 3, 4, 5, 7, 8, 10	RFLP	Mapmaker-QTL V.1.0	[105]
	Anther culture response	DH, synthetic	2, 4, 6	allozyme, isozyme	Segregation distortion	[106]
	embryo formation, callus formation, regeneration, androgenetic factors	RIL, DH	1, 2, 3, 4, 6, 8, 10	RFLPs	Segregation distortion	[107]
	callus induction, plant regeneration	RIL	2, 3, 5, 6, 8, 9	SSR	Composite interval mapping	[108]
	callus initiation, callus formation (totipotency)	RIL	1.05, 1.06, 1.08, 2.04, 2.05, 2.06, 3.06, 4.01, 5.03, 5.04, 6.05, 8.06, 9.03	RFLP, SSR	PlabQTL with cofactor selection	[109]
	culturability, transformability	Backcross population	1.03, 1.04, 1.05, 1.08, 1.09, 1.1, 2.08, 3.05, 3.06, 3.07, 3.08, 6.02, 6.03, 6.04, 10.06, 10.07	RFLP, SSR, SNP	Segregation distortion	[110]
	embryo culturing capacity	$F_{2:3:4}$	1, 3, 7, 8	SSRs	Mapmaker V.3.0b, QTL Cartographer (CIM)	[111]
Rice	regeneration ability	backcross	1, 2, 4	RFLP	ANOVA (GLM), Mapmaker	[112,113]
	callus induction, green plantlet, albino plantlet	DH	1, 6, 7, 8, 10, 12	RFLPs	Mapmaker	[114]

	callus induction, plant regeneration	RIL	1, 2, 3, 11	RFLP, AFLP, SSLP, isozyme, morphological markers	Interval mapping	[115-118]
	shoot regeneration	F_2	2, 4	RFLP	ANOVA, interval mapping (MAPL)	[119]
	regeneration	backcross population	1, 2, 3, 6	PCR markers	QGENE	[94]
Soybean	somatic embryogensis	RIL	group 8(D1b+W), group 6(C2)	point markers	Composite interval mapping	[120]
	somatic embryogensis	RIL	1, 2, 3	SSR	ANOVA	[121]
	callus induction, somatic embryogenesis ability	RIL	B2, D2, G	SSR, EST-SSR	Composite interval mapping	[122]
Sunflower	total embryogenic explants, embryos per total explant	RIL	Linkage groups 1, 3, 4, 6, 11, 13, 15, 16, 17	AFLP	CIM QTL cartographer v1.13	[123]
Sunflower	somatic embryogensis	RIL	Linkage group 5, 10, 13	AFLP, SSR	Mapmaker/Exp 3.0	[124]
Tomato	regeneration	F_2, BC1	1, 3, 4, 7, 8	SSR, COSI, COSII, CAPS, AFLP	Interval mapping, restricted multiple QTL mapping	[125]
Wheat	green spot initiation, shoot regeneration	RIL	2B	RFLP	ANOVA	[126]
	regeneration	DH crosses, bulked segregant analysis	2AL, 2BL, 2AL, 2BL, 5BL	AFLP, microsatellites	Composite interval mapping (PlabQTL)	[127]
Winter rye	percentage of immature embryos/inflorescences, percentage of explants producing somatic embryos	RIL	1R, 4R, 5R, 6R, 7R	SSRs, ISSRs, SAMPLs, RAPDs, EST, SCAR	Mapmaker	[128,129]

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CHAPTER 2: WHOLE TRANSCRIPTIOME PROFILING OF MAIZE DURING EARLY SOMATIC EMBRYOGENESIS REVEALS ALTERED EXPRESSION OF STRESS FACTORS AND EMBRYOGENESIS-RELATED GENES

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2.1 ABSTRACT

Embryogenic tissue culture systems are utilized in propagation and genetic engineering of crop plants, but applications are limited by genotype-dependent culture response. To date, few genes necessary for embryogenic callus formation have been identified or characterized. The goal of this research was to enhance our understanding of gene expression during maize embryogenic tissue culture initiation. In this study, we highlight the expression of candidate genes that have been previously regarded in the literature as having important roles in somatic embryogenesis. We utilized RNA based sequencing (RNA-seq) to characterize the transcriptome of immature embryo explants of the highly embryogenic and regenerable maize genotype A188 at 0, 24, 36, 48, and 72 hours after placement of explants on tissue culture initiation medium. Genes annotated as functioning in stress response, such as glutathione-S-transferases and germin-like proteins, and genes involved with hormone transport, such as PINFORMED, increased in expression over 8-fold in the study. Maize genes with high sequence similarity to genes previously described in the initiation of embryogenic cultures, such as transcription factors BABY BOOM, LEAFY COTYLEDON, and AGAMOUS, and important receptor-like kinases such as SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASES and CLAVATA, were also expressed in this time course study. By combining results from whole genome transcriptome analysis with an in depth review of key genes that play a role in the onset of embryogenesis, we propose a model of coordinated expression of somatic embryogenesis-related genes, providing an improved understanding of genomic factors involved in the early steps of embryogenic culture initiation in maize and other plant species.

2.2 INTRODUCTION

In order to meet global food, feed, and fiber needs in the face of climate change and predicted population growth, current and future crop improvement efforts will likely include the utilization of biotechnology-based approaches [1,2]. This includes the discovery and functional analysis of agriculturally important genes for crop research and product development. Currently, most of the crop genetic engineering systems utilize embryogenic, regenerable tissue cultures as a critical part of the transformation process [3]. Totipotent, embryogenic cultures are also desirable for efficient somatic embryo production for other agricultural biotechnology applications such as clonal propagation, production of synthetic seed [4], and the proposed utilization of somatic embryos for gamete cycling in rapid breeding [5].

At the molecular level, it is widely accepted that the induction of somatic embryogenesis involves massive cellular reprogramming and activation of various signaling cascades [6,7]. The necessary triggers that induce somatic embryogenesis in tissue culture are tantamount with stress response [8,9]. As the accumulation of near-damaging cellular signals trigger change, only specific genotypes are capable of efficient cellular adaptation, pluripotency, and embryogenic competence in tissue culture [10].

The majority of crop genotypes within species display low embryogenic growth response in culture. This genotype-dependent culture response decreases the efficiency and significantly limits the application of clonal propagation schemes and current transformation systems in the genetic study and improvement of crop plants [11]. In maize, the inbred line A188, which displays a high embryogenic culture response, has been utilized in investigations on the inheritance and genetic control of the genotype-dependent culture response [12-14] and in improving embryogenic response efficiency and regeneration ability in tissue culture [15,16].

Despite the agronomic importance, few genes with a direct role in the induction of somatic embryogenesis in tissue culture have been identified, and their role in embryogenic culture response in maize and other crops is not understood. In *Brassica napus*, Arabidopsis, and Chinese white poplar, the transcription factor, BABY BOOM, when ectopically expressed in recalcitrant lines in tissue culture, was shown to induce somatic embryogenesis [6,7]. LEAFY COTYLEDON and PINFORMED genes have been thoroughly studied in zygotic embryogenesis in normal seed development with some studies suggesting that these genes may also be important to somatic embryogenesis in tissue culture [7]. In addition, regulatory genes such as AGAMOUS, WUSCHEL and CLAVATA have been studied in Arabidopsis for their role in meristem formation, somatic embryo formation, and callus maintenance [6,7], yet their role in maize tissue culture is not well understood.

To improve the understanding of genomic factors involved in early somatic embryogenesis in maize, we examined the transcriptome of the highly embryogenic maize inbred line A188 at 0 to 72 hours (h) after placement of immature embryo explant tissues onto culture initiation medium. Some of the first embryogenesis-related alterations in cell processes and cell division that are necessary for efficient embryogenic response occur during the early initiation stages. Based on our findings, we propose a coordinated expression model for somatic embryogenesis-related genes and describe an overview of global expression trends highlighting genes that are up- and down-regulated during the time course of the study. Genes related to somatic embryogenesis in other species and the relative expression of maize genes with high sequence similarity is also discussed. This research provides important information relating to the improvement of crop tissue culture and genetic engineering systems.

2.3 MATERIALS AND METHODS

Plant material and tissue culture initiation

Field grown donor plants were grown at the West Madison Agricultural Research Station (Madison, WI). Immature maize embryos from two plants of the maize inbred line A188 were isolated and cultured as previously described [17] with minor modifications. Briefly, ten days after pollination, 125 immature embryos (1.0 - 1.2 mm from scutellar tip to base) from each of two maize ears were harvested, aseptically dissected from kernels, and then placed onto culture initiation medium by placing embryos axis side down (scutellum side up) on modified N6 tissue culture medium [18]. The medium was prepared with N6-basal salts [18] at 3.98 g/L (PhytoTechnolgies Lab, product number M524), 2 mL/L of 1mg/mL 2, 4-D stock, 2.875 g/L L-proline, 30 g/L sucrose, 3.5 g/L gelzan, pH to 5.8. After autoclaving, filter sterilized N6 vitamins stock (1,000x solution) and silver nitrate stock solution prepared as per protocol [17] were added. Prior to embryo isolation, ears were surface sterilized in a 50% commercial bleach (8.25% sodium hypochlorite) solution with a drop of Tween 20, and then rinsed 3 times in sterile, deionized water. A total of 210 embryos from each of two donor plants, or two biological replicates, were used for this study. Ten to 25 embryos were harvested for the each of 0, 24, 36, and 48h time points. Ten embryos from only one plant, or one biological sample, were harvested for the 72h time point. For the first time point (0h), the embryos were aseptically dissected from kernels and immediately placed into liquid nitrogen without placement on culture medium. For subsequent time points at 24h, 36h, 48h, and 72h after plating, embryos were aseptically isolated and placed onto culture initiation medium.

RNA-seq Library Construction and Sequencing

RNA was extracted using the Invitrogen TRIzol reagent according to the manufacturer's instructions (Invitrogen, http://www.invitrogen.com). Samples were processed using the RNeasy MinElute Cleanup kit (Qiagen, http://www.qiagen.com). RNA quality was assessed using the Agilent RNA 6000 Pico Kit Bioanalyzer prior to preparation of the sequence library. Approximately 5 µg of total RNA was processed for mRNA isolation, fragmented, converted to cDNA, and PCR amplified according to the Illumina TruSeq RNA Sample Prep Kit as per the provided protocol, and sequenced on an Illumina HiSeq 2000 (San Diego, CA) at the University of Wisconsin Biotechnology Center (Madison, WI). Two technical sequencing replicates were conducted for each of the two biological sample collections for the 0, 24, 36, and 48h time points and one biological sample for the 72h time point, each with 101 nucleotide single-end reads. Sequences are available in the Sequence Read Archive at the National Center for Biotechnology Information (BioProject accession number PRJNA242658). Sequence quality for each sample was evaluated using the FastQC software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and all samples passed quality control analysis. For subsequent analyses, FPKM values from the two technical sequencing replicates were averaged to represent transcript abundance for each time point 0, 23, 36, and 48h. FPKM values from two technical sequencing replicates were averaged from one biological sample for time point 72h.

Data Analysis

To quantify transcript abundance, sequence reads for each sample were mapped to the maize v2 pseudomolecules (AGPv2; http://ftp.maizesequence.org) [19] and 8,681 non-RTAs that were assembled using RNA-seq reads from 503 diverse maize inbred lines [20]. Mapping was performed using Bowtie version 0.12.7 [21] and TopHat version 1.4.1 [22] with a minimum and maximum intron length of 5 bp and 60,000 bp respectively and the no-novel-indels option. All

other parameters were set to the default values. Normalized gene expression levels were determined using Cufflinks version 1.3.0 [23] setting a maximum intron size of 60,000 bp, the version 5b annotation (http://ftp.maizesequence.org) as the reference annotation, and the AGPv2 fasta sequences for the bias detection and correction algorithm. All other parameters were set to the default values. Pearson's correlation of transcript abundance estimates were measured between biological replicates. Transcripts for samples for 0, 24, 36, and 48h time points were averaged between the two biological replicate samples while transcripts for the 72h time point represented transcripts detected in only one biological sample.

K-means clusters were determined using uncentered Pearson's correlation coefficients in DNA Star ArrayStar version 5.1.0 build 114 allowing 6 clusters and 100 iterations. Only genes with an FPKM value greater than zero at any given time point were included. For an analysis of differential gene expression, each time point was compared to the control time at 0h. A threshold for differential expression of greater than 8-fold for raw FPKM values was used. In order to include genes that may have not been expressed at any given time point but then showed expression at other time points, we included genes with a sum of 2 FPKM or greater in the differential gene expression analysis. Raw values were log2 transformed and visualized on a scatter plot in DNA Star ArrayStar version 5.1.0 build 114. In order to determine coexpression of selected genes, an analysis was done in the R using the xtable statistical computing package version 3.0.2 to calculate the Pearson correlation where the minimum coefficient was set to a threshold of 0.75. In the discussion highlighting the coexpression of specific somatic embryogenesis-related genes, the threshold was set to 0.90.

Gene ontology enrichment analysis was conducted in the PlantGSEA database (http://structuralbiology.cau.edu.cn/PlantGSEA/index.php) [24] to describe groups of genes in

specific clusters or groups of genes differentially expressed with large fold expression changes in different time point comparisons. Enrichment analysis determined maize gene sets that characterized each group as determined by statistical analysis. Fisher's exact test took into account the number of genes in the group query, the total number of genes in a gene set, and the number of overlapping genes. A multiple test false discovery rate correction using the Yekutieli method was set to a cutoff *P*-value at 0.05. Additional annotations were determined by MapMan genome release for *Zea mays* based on B73 5b filtered gene sets (http://mapman.gabipd.org/) [25].

Maize sequence similarity

Maize genes with high sequence similarity to somatic embryogenesis related genes were determined by comparing the maize 5b.60 protein sequences to the protein sequence of previously cloned and characterized genes using BLASTP in the MaizeGDB BLAST POPcorn Project Portal (http://popcorn.maizegdb.org/main/index.php). Input parameters were set to an e-value cutoff of 1e-4 and the maximum number of hits was set to 500. Maize genes with a percent identity greater than or equal to 50% were analyzed for the presence of the conserved binding domain or other features specific to the gene of interest in the National Center for Biotechnology Information (NCBI) Batch Web CD-Search Tool (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) and NCBI Conserved Domains CD-Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to determine genes with the best match. Sequence similarity reported in this study by pairwise alignment was done in LALIGN (http://embnet.vital-it.ch/software/LALIGN_form.html) as the percent identity by local or global alignment.

2.4 RESULTS

GLOBAL ANALYSIS

RNA-seq reads were generated for nine immature embryo samples consisting of 25 embryos per sample of the maize inbred line A188. Embryo samples were placed on culture initiation medium from 0 to 72 hours. In total, the number of reads per sample ranged from 11 million (M) to 36M (Supplemental Table 1). Reads were aligned to the B73 maize reference genome sequence [19] and a set of representative transcript assemblies (RTAs) missing in the B73 reference genome sequence that were identified in transcriptome analyses of 503 maize inbred lines including B73 [20]. Expression values were determined using fragments per kb exon model per million mapped reads (FPKMs) using Cufflinks [23]. Biological replicates at each time point were correlated to assess data reproducibility. Pair-wise Pearson's correlations of expression values between embryos from two different donor plants collected at the same time point ranged from 0.9643 to 0.9927 (Supplemental Table 2), indicating a high degree of reproducibility. Based on this analysis, average expression values from the two replicates were used for downstream analyses. A total of 28,992 annotated B73 reference genes and 6,405 RTAs were expressed in at least one time point (FPKM > 0); while 10,464 reference genes and 2,276 RTAs were not expressed in any sample.

The highest expressed reference genes across time points included genes that function in stress response, RNA binding, DNA synthesis and chromatin structure (Table 1). For example, GRMZM2G156632 is a highly expressed gene which is annotated as wound induced protein 1 (WIP1). Another gene related to plant defense that was among the highest expressed genes was GRMZM2G051943, which encodes for chitinase A1. The RTA with the highest expression at 0 h was joint_Locus_12721 with an FPKM value of 399.89 which decreased over 8-fold to 49.13 at 72 h. The highest RTA expressed at 36 and 48 h was joint_Locus_33043 with an FPKM value

of 13.07 at 0 h and FPKM values of 309.83 and 200.60 at 36 and 48 h, respectively. This RTA was annotated as encoding IN2-1, which based on sequence similarity, is a glutathione S-transferase (GST) protein. The highest expressed RTA at 72 h was joint_Locus_83 (204.13 FPKM) which did not match any known gene annotations.

Characterization of genes with 8-fold or greater expression change

In order to gain an understanding of genes expressed in this time course study, we selected genes differentially expressed by at least 8-fold compared to the control time point (0 h). Comparison of gene expression patterns across the surveyed time points indicated that the largest number of genes with a change in expression profile was from 0 to 24 h (Figure 1). This is supported by the observation that 1,856 genes were expressed at (or greater than) an 8-fold change when comparing 0 vs 24 h, 1,559 genes at an 8-fold change when comparing 0 vs 36 h, 1,496 genes at an 8-fold change when comparing 0 vs 48h, and 1,488 genes at an 8-fold change when comparing 0 vs 72 h. Similarly, comparisons at other time points revealed 177, 45, and 41 genes differentially expressed 8-fold in comparisons of 24 vs 36, 36 vs 48, and 48 vs 72h, respectively. Most genes differentially expressed at 8-fold were up-regulated. For example, 72%, 67%, 66%, and 72% of the genes differentially expressed when compared to 0h were up-regulated at 24, 36, 48, and 72 h time points, respectively. When considering a 2-fold change in expression, 8,174 genes were differentially expressed when comparing 0 vs 24 h, 6,737 genes when comparing 0 vs 36 h, 6,444 genes when comparing 0 vs 48 h, and 6,580 when comparing 0 vs 72 h.

The most abundant genes with large expression changes were enriched for biological processes such as oxidation-reduction processes, metabolic processes, protein phosphorylation, and transmembrane transport. For example, genes with an 8-fold expression change or greater at

24 h were enriched for antiporter and transmembrane transport such as GRMZM2G006894, a hydrogen-exporting ATPase and GRMZM2G479906, GRMZM2G415529, and GRMZM2G366146 are ABC transporters. Other genes up-regulated at least 8-fold at 24 h were involved in transport of amino acids, sugars, and peptides or were specific to transmembrane transport of important nutrients. There were fewer genes that were down-regulated at 8-fold or greater. These genes were involved in membrane transport of amino acids and metals. For example, GRMZM2G140328 and GRMZM5G892495 are down-regulated 8-fold at 24 h and are both involved in calcium signaling. Genes that were up-regulated 8-fold or greater at 72 h revealed glycosyl-related genes such as GRMZM2G179063, a UDP-glucosyltransferase, and iron ion binding genes such as GRMZM2G103773, a BRASSINOSTEROID-6-OXIDASE 2. Genes down-regulated greater or equal to 8-fold between 0 h and 72 h were also enriched for genes involved in stress response such as ATP binding heat shock proteins GRMZM2G360681 and GRMZM2G310431, genes involved in nutrient assimilation such as GRMZM2G087254 and AC189750.4_FG004 both adenylyl-sulfate reductases, and genes involved in regulation of transcription such as GRMZM2G011789, a CCAAT box binding transcription factor.

Characterization of genes grouped by k-means analysis

The induction of somatic embryogenesis involves a complex coordination of multiple pathways [26,27]. Genes involved in hormone response, signal transduction, stress response, transcriptional regulation and cellular reorganization have been described previously [7,9,26]. We sought to determine if our maize transcriptome data supported concepts and models regarding these major biological functions during the very early stages of embryogenic tissue culture initiation. Using k-means clustering with six clusters, we identified groups of genes with similar expression patterns including: (1) up-regulated and then down-regulated during the

developmental window highlighted in this study, (2) both up- and down-regulated during the time course, (3) genes with an expression trend towards increased up-regulation from 0 to 24 h, (4) genes with a higher up-regulation later in the time course at 36, 48 and 72 h compared to all other genes expressed in the developmental window, (5) genes with an expression trend towards large-scale down-regulation from 0 to 24 h, and (6) genes with constitutive expression throughout the study (Figure 2).

Gene ontology enrichment was significant for clusters 1, 2, 3, and 6. Genes in cluster 1 were enriched for protein kinase and phosphorylation activity. Specifically, these genes were enriched for functions involving DUF26 signaling receptor kinases and post-translational modification receptor like kinases, as well as UDP glucosyl and glucoronyl transferases. Gene expression values in cluster 2, enriched for apopotic processes, ranged from a minimum FPKM of 0.006 to a maximum of 4.414. Since the expression of these genes in cluster 2 was very low, these FPKM values could be inaccurate and attributed to noise. Genes in cluster 3 (initially up-regulated) were involved in numerous functions including transmembrane transport activity, oxidation-reduction processes, and heme binding or iron ion binding such as cytochrome P450 related genes. Finally, genes in cluster 6 were enriched for intracellular functions such as chromatin structure and DNA synthesis, ribosomal proteins synthesis, transcription factors, cell transport and RNA processing. A total of 2,704 RTAs, the largest proportion of RTAs grouped into a k-means cluster, were grouped into cluster 6.

Since gene ontology enrichment was not significant for clusters 4 and 5, MapMan B73 5b gene annotations [25] were used to describe genes in these clusters. Genes in cluster 4 were related to protein degradation, signaling receptor kinases, transcription factors, and genes involved in hormone metabolism and secondary metabolism. Genes in cluster 5 were involved in

similar functions to cluster 4, but in addition, some cluster 5 genes were annotated for functions in amino acid and lipid metabolism. RTAs with annotations relating to transcription factors that promote embryo development which could be involved in somatic embryogenesis are RTA joint_Locus_9393 annotated as an ethylene responsive transcription factor and expressed from 32.70 to 22.46 at 0 h and 72 h, respectively. Similarly, the RTA joint_Locus_7247 which was annotated as encoding an AP2 domain transcription factor was expressed from 10.01 to 14.33 at 0 and 72 h, respectively. Both RTAs were grouped into cluster 6 by k-means analysis. RTAs with interesting annotations and expression trends related to stress factors are joint_Locus_19099 annotated as encoding a GST-30 which showed a decrease in expression from 60.98 at 0 h to 5.41 at 72 h, and joint_Locus_9459, annotated as a cytochrome P450 in maize, which showed an increase in expression from 1.82 at 0 h to 22.04 at 24 h.

Candidate genes previously described in somatic embryogenesis

We performed an in-depth review of the literature to identify major candidate genes previously reported or suggested to be important for somatic embryogenesis in maize and other species, and using sequence similarity we identified orthologs in maize for genes identified in other species (Table 2).

Genes involved in stress responses previously suggested to be important in somatic embryogenesis include GST and germin like-proteins (GLP). Using gene accessions [28] and protein sequence similarity, we identified 15 maize GST genes, of which several showed an 8-fold or greater increase from 0 to 24 h (Table 3). In addition, one maize GLP gene GRMZM2G045809, annotated as ZmGLP2-1 [29], was up-regulated greater than 8-fold from 1.44 at 0 h to 251.27 FPKM at 72 h (Table 3). These stress response genes exhibiting a large fold change and increased expression from 0 to 24 h were grouped into k-means cluster 3.

Genes involved in embryogenic pathway initiations include BABY BOOM (BBM) and LEAFY COTYLEDON (LEC) genes [7]. In this study, we highlight three maize genes that showed high sequence similarity to the highly conserved AP2 binding domain of *Brassica napus* BBM (BnBBM1, accession number AF317904). GRMZM2G366434, GRMZM2G141638, and GRMZM2G139082 are 91.2%, 92.5%, and 93.2% similar to the translated amino acid sequence of BnBBM1, respectively (Supplemental Figure 1). GRMZM2G366434 showed a 4-fold up-regulation relative to 0 h at 36, 48 and 72 h (Figure 3A), GRMZM2G141638 also increased during this time course (Figure 3B), and GRMZM2G139082 increased over 4-fold from 0 to 72 h (Figure 3C). These maize BBM-like genes were grouped into cluster 3.

GRMZM2G011789, the maize ortholog to LEAFY COTYLEDON1 (ZmLEC1), was grouped into cluster 5 by k-means analysis. GRMZM2G011789 was expressed early initially (62.07 FPKM at 0 h) and then decreased dramatically to 2.90 at 24 h and 0.56 at 72 h (Figure 3D). Using sequence similarity, we found that maize gene GRMZM2G405699 is 47.4% similar in protein sequence to the Arabidopsis LEAFY COTYLEDON2 (AtLEC2, Supplemental Figure 2) and 99.8% similar to the maize VIVIPARIOUS1 (VP1) gene GRMZM2G133398 (GenBank accession M60214). GRMZM2G405699 showed a moderate increase in expression during the time course of this study from 23.05 FPKM at 0 h to 30.50 FPKM at 72 h (Figure 3E), and GRMZM2G133398 (VP1) showed a different expression pattern with a moderate decrease from 59.04 FPKM at 0h to 46.03 FPKM at 72 h (Figure 3F). GRMZM2G405699 and GRMZM2G133398 were grouped into k-means cluster 6.

SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) genes are also important for embryogenic pathway initiation. In this study, expression of SERK1 (ZmSERK1, GRMZM5G870959) was minimal, ranging from 4.23 to 5.86 FPKM. Similarly, the orthologs to

maize SERK2 (ZmSERK2, GRMZM2G115420) and the ortholog to maize SERK3 (ZmSERK3, GRMZM2G150024) showed very similar magnitudes in expression and trend increasing from about 15 to 20 FPKM. In our study, both ZmSERK2 and ZmSERK3 increased nearly 2-fold from 0 h to 24 h. Maize SERK genes were grouped into cluster 6.

PIN1 is involved in auxin transport [30]. The maize PINFORMED1 (PIN1) gene, (ZmPIN1a, GRMZM2G098643) displayed up-regulation with FPKM values of 11.02 at 0 h to 153.59 at 72 h (Figure 4A), and additional orthologs to maize PINFORMED1 (ZmPIN1b, GRMZM2G074267) and (ZmPIN1c, GRMZM2G149184) also increased in expression. ZmPIN1a and ZmPIN1b were grouped into cluster 6; ZmPIN1c was grouped into cluster 3 (Figure 2).

Known genes involved in embryo formation and development include WUSCHEL, CLAVATA, AGAMOUS and WOX genes. ZmWUS1 (GRMZM2G010929) was minimally expressed, not exceeding 1 FPKM during this time course (Figure 4B), and ZmWUS2 (GRMZM2G028622) was not expressed in any sample. GRMZM2G14151 has high sequence similarity to the CLAVATA (CLV1) gene in Arabidopsis (Supplemental Figure 3) and increased in expression from 13.14 FPKM at 0 h to 20.06 FPKM at 72 h (Figure 4C). GRMZM2G14151 was grouped into k-means cluster 6. Maize genes that are orthologs to AGAMOUS, which include ZMM2 (GRMZM2G359952), ZAG1 (GRMZM2G052890), and ZAG2 (GRMZM2G160687), showed minimal expression during the time course of this study (Figure 4D and Table 2). A BLAST search for AGL15 revealed a number of maize genes with high sequence similarity. For example, ZmMADS69 (GRMZM2G171650), ZmMADS52 (GRMZM2G446426), and ZmMADS73 (GRMZM2G046885) show 67.74%, 64.41%, and 42.11% sequence similarity to the AGL15 amino acid sequence in Arabidopsis. These MADS

box transcription factors were grouped into k-means cluster 6 and were moderately expressed throughout the time course, with FPKM values greater than 10 at every time point (Supplemental Figure 4A-C). One maize gene, ZmMADS11 (GRMZM2G139073), has 45.75% sequence similarity to AGL15. ZmMADS11 was grouped into k-means cluster 3 and was shown to have an 8-fold expression change at each time point compared to 0 h (Supplemental Figure 4D). We also examined the expression of maize WUSCHEL-related homeobox domain (WOX) genes and found ZmWOX2A (GRMZM2G108933), ZmWOX5A (GRMZM2G478396), ZmWOX5B (GRMZM2G116063), and ZmWOX11/12B (GRMZM2G314064) showed an 8-fold increase in expression after placement of immature embryos into the tissue culture environment and grouped into k-means clusters 1, 2, and 3 while other maize WOX genes were grouped into clusters 5 and 6 (Table 4).

2.5 DISCUSSION

Somatic embryogenesis-related genes have been extensively characterized in Arabidopsis; however, relatively few have been evaluated in maize. Using transcriptome data of maize in embryogenic tissue culture initiation, this study provides an in-depth look at the major candidate genes discussed in previous reviews and research studies on somatic embryogenesis. Moreover, we propose a model (Figure 5) based on coordinated expression of somatic embryogenesis-related genes highlighted in this study and their relative expression in early embryogenic tissue culture response.

Genes associated with stress response in tissue culture

Our observations support previous reviews on the transition to somatic embryogenesis, with our whole transcriptome data showing a large number of genes expressed during the early

stages of somatic embryogenesis from 0 h to 24 h in tissue culture. Gene enrichment analysis of genes clustered based on k-means, and of genes grouped by large fold changes when compared to the control time point 0h, supported major biological functions suggested in previous studies as important for somatic embryogenesis such as stress response, transmembrane transport, and hormone metabolism [6,8,9,27]. Genes with a large fold change and genes grouped in cluster 3 in this study include cytochrome P450, UDP-glucosyl, and glucoronyl transferases. In another study involving an embryogenic maize line from China, genes differentially expressed in the early stages of embryogenesis were also related to stress where metabolism of xenobiotics by cytochrome P450 was identified as one of the most significant pathways by enrichment analysis of differentially expressed genes in samples grown 1-5 days after tissue culture [31].

In this study, we identified two maize genes, WIP1 (GRMZM2G156632) and chitinase A1 (GRMZM2G051943) which were up-regulated over 1500-fold from 0 h to 24 h. These genes have been previously described in plant defense and stress response [32,33], but have not, until now, been associated with tissue culture response in maize. WIP1 has previously been characterized as a defense gene based on its involvement in hypersensitive defense response [33]. Chitinase proteins have been suggested to promote somatic embryogenesis [9] since one study in carrot showed that a non-embryogenic mutant line was triggered to produce somatic embryos after the addition of chitinase proteins in the tissue culture medium [34].

GSTs are a family of genes also involved in plant defense [30] and we observed 15 out of the 33 maize GST genes with large fold expression changes during early somatic embryogenesis (Table 3). It has been suggested that some GSTs may function in tissue dedifferentiation by affecting the cell's redox status by changing endogenous levels of important plant growth hormones such as auxin [6]. GSTs were also detected in chicory during somatic embryogenesis

in callus cultures initiated with leaf tissue [35], and GSTs were also expressed in response to auxin treatment in *Cyclamen persiucum* tissue culture with an initial up-regulation during the first 4 hours followed by down-regulation at 72 h [36]. In this study, GST genes were found to be coexpressed with BBM, WUS, PIN, and SERK genes (Figure 5). We also detected one maize GLP gene (GRMZM2G045809) with a large fold change in expression at 72 h (Table 3). Moreover, this GLP gene was shown to be coexpressed with the BBM transcription factor (Figure 5). GLPs are proteins that also affect the plant redox status and are involved in developmental regulation. In wheat embryogenic callus cultures, GLPs were detected as early as 2 to 72 hours after plating explant tissues in culture [37]. GLPs are typically detected in embryogenic tissues, but not in non-embryogenic tissues. GLPs with superoxide dismutase activity promote the production of hydrogen peroxide (H_2O_2), a type of oxidative stress. It has been suggested that the H_2O_2 produced may serve as a secondary signaling molecule acting to promote somatic embryogenesis [8,9].

Genes involved in embryogenic pathway initiation

Embryogenic pathway initiation is marked when somatic cells acquire embryogenic competence and proliferate as embryogenic cells capable of forming somatic embryos [8]. One gene that has been attributed to initiation of somatic embryogenesis across plant species is BBM. BBM genes highlighted in this study were found to be coexpressed with GLP, GST, PIN, WOX, LEC2, and AGL15 genes (Figure 5). BBM was first discovered in investigations of *Brassica napus* microsporogenesis by subtractive hybridization [38]. The gene was consistently expressed only in embryogenic microspore cultures. Sequence analysis showed that BBM has two unique binding domains: an APETALA-like AP2 binding domain and an ethylene-responsive element binding factor, both characteristic of functioning in plant hormone signaling and regulation [38].

Overexpression of BBM in Arabidopsis and *B. napus* led to the induction of somatic embryogenesis and regeneration ability without the addition of exogenous plant hormones [38]. This observation suggested that BBM acts as a stimulator of plant hormone production, triggering signaling pathways important for somatic embryogenesis [38,39]. Overexpression of BBM-induced embryo formation enhanced regeneration ability in Chinese white poplar [40] and tobacco, [41] and improved transformation efficiency in sweet pepper [42]. In another study focused on transforming artificial chromosomes into maize, the shuttle vector used contained a BBM homolog called ZmODP2 to promote cell division and callus growth after transformation [43]. Researchers suggested that the presence of this construct improved transformation efficiency in maize tissue culture by 20-50%. In our study, we identified three maize genes GRMZM2G366434, GRMZM2G141638, and GRMZM2G139082 with high sequence similarity to BnBBM1 and which contain the conserved and unique AP2 binding domain (Supplemental Figure 1). These maize genes were also shown to increase in expression during early somatic embryogenesis (Figure 3) in this study. When we compared these maize gene expression trends to transcripts detected in the maize B73 gene atlas [44], expression was not at all detected (0 FPKM) in whole seeds or endosperm at 10, 12, 14, and 16 days after pollination. Expression was, however, detected in zygotic embryos 16 days after pollination, in germinating seed, in the primary root and in V3 stem and shoot apical meristem [45].

Another important group of genes involved in embryogenic pathway initiation are the LEC genes. LECs are transcription factors identified in studies of zygotic embryogenesis in plants that have been proposed to be important for somatic embryogenesis [7,46]. Mutational analysis of LEC genes showed their function in early zygotic embryogenesis, specifically, to maintain suspensor cell fate and specify cotyledon identity [47]. LEC genes play an important

regulatory role directly interacting with hormone response genes [48,49]. AtLEC1 was cloned and ectopically expressed in transgenic Arabidopsis seeds, showing its essential role in germination and embryonic organ identity [50]. AtLEC1 in Arabidopsis in tissue culture was also shown to be differentially expressed in embryogenic compared to non-embryogenic samples [51]. One study on the highly embryogenic maize hybrid HiII, ZmLEC1 transcription in somatic embryos showed a high initial expression and then a decrease in expression during early development of [52]. We found a similar result in our study, where ZmLEC1 (GRMZM2G011789) decreased in expression over 20 fold during the first 24 hours in tissue culture. In contrast, the expression of a maize gene similar to AtLEC2 (GRMZM2G405699) based on high sequence similarity (Supplemental Figure 2) increased steadily in this study (Figure 3), was grouped into k-means cluster 6, and is coexpressed with BBM (Figure 5). The role for AtLEC2 in Arabidopsis zygotic embryogenesis to induce somatic embryos by activating auxin responsive genes was proven by ectopic expression [53,54]. AtLEC2 is nearly identical to VP1 in Arabidopsis [7,54] and in this study, we found that the maize gene GRMZM2G405699, which is most similar to AtLEC2, is also highly similar to maize VP1 (GRMZM2G133398). Both AtLEC2 and VP1 genes share the same class of unique B3 domains. One study involving gene expression analysis on T-DNA insertion lines in Arabidopsis suggested a role for VP1-like genes in recruiting chromatin-remodeling factors that can either activate or repress LEC1-like activity during seed development [55]. Moreover, it has been suggested that this complex network involving LEC1 and LEC2 genes in seed development can up-regulate important transcription factors such as BBM during early zygotic embryogenesis in Arabidopsis [49]. Our study showed GRMZM2G405699 coexpressed with maize BBM-like genes during early somatic embryogenesis (Figure 5).

PIN1 genes encode influx and efflux carrier proteins that mediate auxin transport in early zygotic embryo development [56]. In this study, PIN1 was coexpressed with GST, BBM, and WOX genes (Figure 5). In Arabidopsis, PIN genes, are essential for embryonic stem cell growth [57] and are expressed in early proembryonic development [58]. In maize, PIN1 genes also play a role in auxin transport and tissue differentiation during zygotic embryogenesis [59]. The ZmPIN1a gene was highly expressed in this study (Figure 4A), and increased dramatically from the 24 to 72h time point. A study using *in situ* hybridization of ZmPIN1a and ZmPIN1b showed transcript abundance and protein localization of PIN proteins in maize kernels, endosperm and embryo [59,60]. The authors also suggested a role for PIN1 in maize development during zygotic embryogenesis in mediating polar auxin transport and patterning during development [30,59]. In tissue culture, an important step in establishing embryogenic patterning in embryos is apical-basal rearrangement [51]. Our observations also show that PIN1 genes are expressed during tissue culture response in early somatic embryogenesis.

Genes involved in somatic embryo formation and development

There is evidence that genes involved in meristem formation are also important in somatic embryo formation. For example, WUS is a homeodomain transcription factor involved in shoot and floral meristem development specifically as a regulator of stem cell fate and organ identity [61]. WUS expression has been detected in a small group of cells described as the organizing center of meristematic tissue. This organizing center is localized underneath a larger mass of stem cells [57,62,63]. WUS has an important role in regulating and activating pluripotent stem cells by promoting proliferation genes and repressing developmental regulators [64]. While, it has been shown in Arabidopsis that PIN1-mediated auxin transport directly

induces WUS expression in early somatic embryogenesis [57], in our study, ZmWUS genes were minimally expressed, however, were coexpressed with GST and SERK genes (Figure 5). We hypothesize that the developmental window highlighted in this study may have captured a time when the organizing center was just initiating in development and that transcripts detected represented few cells showing WUS activity during the early stages of stem cell development. In addition, it is plausible that suitable endogenous auxin concentrations were just beginning to establish. Over time, more cells either localized in or on the organizing center would also display WUS transcriptional activity.

Detailed analysis of the expression pattern of WUS orthologs in maize and rice showed that WUS genes in higher plants did not mimic expression localized in the organizing center as it did in Arabidopsis implying a major modification in plant evolution [65]. Our findings showed that WUS genes had minimal to no expression in early embryogenesis in tissue culture but some maize WOX genes increased in expression over 8-fold. WOX genes were coexpressed with BBM, PIN and AGL15 (Figure 5). WOX expression was detected in somatic embryogenesis in other plants where efficient embryogenic callus cultures are also genotype-dependent [66]. In our study, we highlight a number of maize WOX genes with differential expression compared to time point 0h (Table 4) of which ZmWOX2A, ZmWOX5A/5B, and ZmWOX11/12B showed an 8-fold change in expression. Some examples of WOX genes in tissue culture in other plants include, WOX2 associated with somatic embryogenesis in conifer tissue culture [67] and WOX11 in grapes detected in embryogenic versus non-embryogenic cultivars *in vitro* [66].

Expression of a CLV1-like gene (GRMZM2G141517), however, showed a steady increase in this study (Figure 4C). CLV1 is a receptor-like kinase also involved in shoot and floral meristem development [68] and acts upstream of WUS. CLV1 represses WUS activity by

interacting in a regulatory loop with WUS to promote callus initiation and maintenance [69]. Capturing expression of these genes at later time points in tissue culture would provide insight on transcriptional activity between the regulatory loop between WUS and CLV1 in maize.

Another meristem-related gene discovered in Arabidopsis is AGAMOUS, a MADS box transcription factor involved in flower development and organ differentiation [70]. AGAMOUS had been shown to interact directly with WUS also by repressing its expression in floral stem cells [63,71]. Similar to ZmWUS, genes highly similar to AGAMOUS, such as ZAG1, ZAG2 and ZMM2 were minimally expressed in this study. However, we did observe differential expression relative to 0h for an AGL15-like gene (GRMZM2G139073) (Supplemental Figure 4D). In addition, GLP, PIN, WOX and BBM genes were coexpressed with GRMZM2G139073 (Figure 5). AGL15 in *B. napus* and Arabidopsis embryos [70] have been shown to be preferentially localized in embryonic tissues [72]. Additionally, AGL15 was shown to promote somatic embryo development in Arabidopsis and soybeans [73]. Studies also suggest that AGL15 in Arabidopsis interacts with LEC2 directly [48,74] and, immunoprecipitation and time-of-flight mass spectrometry revealed AGL15 was included in the SERK1 complex *in vivo* [75]. To date, there have been no studies of AGL15 –like genes expressed in maize somatic embryos that have been reported. From studies on AGL15 in Arabidopsis in promoting somatic embryogenesis and interacting with LEC2 and SERK1, we hypothesize that maize AGL15-like genes may also be important for callus initiation and maintenance.

2.6 CONCLUSION

Deciphering the underlying genetic mechanisms controlling somatic embryogenesis in tissue culture is important for improving our understanding of the basic processes involved in somatic embryo formation, and in the development of embryogenic tissue culture systems that

are less genotype dependent. Although few major genes that promote somatic embryogenesis in Arabidopsis and other plants species have been described, even fewer genes have been studied and their expression revealed in the context of the whole transcriptome in tissue cultures of maize. In this study, we highlighted the expression of maize genes with high sequence similarity to BBM, LEC2, CLV1, and AGL15, and maize SERK and PIN genes, and discussed their potential role in somatic embryogenesis. Many of the somatic embryogenesis related genes analyzed in this study fall into a k-means clusters 3 with an expression trend towards an initial large up-regulation and a second cluster number 6, with genes that are moderately to highly expressed throughout the targeted developmental. However, clusters 4 and 5 also show interesting expression trends that could be important for further studies due to their large up- and down-regulation expression trends, respectively. In this investigation, we also highlighted maize gene families, mainly GST, GLP, and WOX genes and identified specific genes within gene families with altered expression. A number of specific genes discussed in this study could be potential candidates for further testing regarding their importance and contribution to embryogenesis in tissue culture in maize.

Whole transcriptome profiling during the very early stages in the initiation of somatic embryogenesis in culture of the highly embryogenic, regenerable maize genotype, A188, now provides new information on the expression of somatic embryogenesis-related genes in maize. By studying the whole transcriptome during a specific developmental window, we were able to provide data on transcripts detected for major genes previously described with a role in embryogenesis. This information can be utilized to help us better understand major gene functions and expression networks involved in the induction of somatic embryogenesis in culture. Investigations involving fine-mapping and identification of specific genes in maize that

confer regeneration ability could build on the findings reported here to further enhance our understanding of which many genes expressed in concert are possible key factors underlying the genotype dependent nature of tissue culture phenotypes. In the same way, a study involving the analysis of the whole transcriptome of isogenic lines differing in their ability to produce embryogenic, regenerable cultures, and their representative transcripts that are not mapped to the reference genome, could also add to identifying causal genes, providing a deeper understanding of the somatic embryogenesis-related genes we described here, and allow determination of their level of significance in the process. Improving our understanding of the biological processes and the genetic mechanisms that confer efficient tissue culture response such as somatic embryogenesis *in vitro* will help crop improvement strategies and functional genomics testing that is necessary to increase agricultural productivity in a changing global agricultural landscape.

Table 2.1. Highly expressed genes in immature zygotic embryo explants of maize inbred line A188 in tissue culture.[a]

		FPKM					
Gene	Function	0 h	24 h	36 h	48 h	72 h	Cluster Number
GRMZM2G020940	unknown	889.44	1548.51	1168.15	1203.65	1282.68	6
GRMZM2G080603	grp1	1005.03	1816.68	2345.42	2365.11	2412.29	6
GRMZM2G480954	unknown	22.07	1217.33	993.33	844.36	571.95	6
GRMZM2G153292	tua2	1292.61	1065.69	1384.88	1349.02	1313.01	6
GRMZM2G080274	ARATH HON1 Group	1205.96	164.49	251.87	293.59	288.91	6
GRMZM2G337229	ole1	1129.77	1341.73	1377.95	1155.93	812.47	6
GRMZM2G051943	chitinase A1	1.01	1636.10	2150.28	1676.96	1331.93	3
GRMZM2G332838	Histone H4	1295.95	267.26	649.95	642.20	566.99	6
GRMZM2G011523	unknown	6.47	1952.69	1191.21	825.81	451.70	3
GRMZM2G057823	ald1	1421.28	802.19	931.20	729.20	695.52	6
GRMZM2G088511	unknown	998.56	928.75	1323.82	1023.23	1005.62	6
GRMZM2G084195	Histone H4	1273.38	358.28	845.45	883.28	770.60	6
GRMZM2G091715	unknown	1207.21	495.31	568.79	534.97	466.14	6
GRMZM2G303374	unknown	954.01	888.49	1058.47	1105.93	1267.46	6
GRMZM2G152466	tua4	1504.67	548.22	923.32	1070.86	1147.94	6
GRMZM2G165901	rab15	1063.15	2870.95	2945.72	2222.61	1919.31	6
GRMZM2G072855	Histone H4	1242.98	248.54	541.83	557.07	509.32	6
AC233865.1_FG001	Histone H4	2001.74	427.13	765.30	812.44	745.99	6
GRMZM2G031545	unknown	801.56	714.73	982.54	1193.72	1366.95	6
GRMZM2G156632	WIP1	2.36	7158.54	2770.89	788.17	254.05	3
GRMZM2G028393	sci1	18.32	4544.08	2155.63	1779.17	1152.94	3
GRMZM2G126900	unknown	1.15	1308.76	571.75	490.23	435.86	3

[a.]Fragments per kilobase of exon per million fragments mapped (FPKM) at 0, 24, 36, 48, and 72 h after placement on tissue culture initiation medium and the assigned gene cluster number determined by k-means analysis.

Table 2.2. Somatic embryogenesis-related genes, National Center for Biotechnology Information (NCBI) accessions, and plant species where accession was previously characterized.

Gene name	NCBI gene accession number	NCBI protein accession number	Species	Reference
AGL15	U22528	AAA65653	Arabidopsis	[70]
AtLEC2	AF400124	AAL12005	Arabidopsis	[53,76]
BnBBM1	AF317904	AAM33800	*B. napus*	[38,39,41,42]
CLV1	U96879	AAB58929	Arabidopsis	[68]
ZAG1	L18924	AAA02933	Maize	[77]
ZAG2	L18925	AAA03024	Maize	[77]
ZmLEC1	AF410176	AAK95562	Maize	[52,78]
ZMM2	L81162	AAB81103	Maize	[79]
ZmPIN1a	DQ836239	ABH09242	Maize	[59,60]
ZmPIN1b	DQ836240	ABH09243	Maize	[59,60]
ZmPIN1c	EU570251	ACB55418	Maize	[59]
ZmSERK1	AJ400868	CAC37640	Maize	[80,81]
ZmSERK2	AJ400869	CAC37641	Maize	[80,81]
ZmSERK3	AJ400870	CAC37642	Maize	[80]
ZmWUS1	AM234744	CAJ84136	Maize	[65]
ZmWUS2	AM234745	CAJ84137	Maize	[65]

Table 2.3. Expression of maize glutathione-S-transferase genes (ZmGST) and maize germin-like proteins (ZmGLP) in tissue culture.[a]

		FPKM						
Gene name	Annotated name	0 h	24 h	36 h	48 h	72 h	Fold change	Cluster number
ZmGST 8	GRMZM2G156877	0.63	25.66	32.87	33.53	24.51	8-fold	3
ZmGST 9	GRMZM2G126763	1.14	0.14	0.34	0.54	0.45	8-fold	2
ZmGST 10	GRMZM2G096153	21.22	17.45	18.86	23.83	24.42		6
ZmGST 11	GRMZM2G119499	1.02	0.92	2.81	7.00	7.15		4
ZmGST 12	GRMZM2G096269	1.04	0.84	2.43	4.76	2.32		4
ZmGST 13	GRMZM2G126781	0	0	0.22	0.65	0		
ZmGST 14	GRMZM2G175134	1.14	2.90	6.02	12.58	15.26	8-fold	3
ZmGST 15	GRMZM2G150474	0.18	3.25	3.85	3.66	2.64	8-fold	3
ZmGST 16	GRMZM5G895383	0	0.13	0.12	0.26	0.43		
ZmGST 18	GRMZM2G019090	7.05	121.54	206.88	175.17	105.79	8-fold	6
ZmGST 19	GRMZM2G335618	0.73	52.51	45.53	50.03	49.92	8-fold	3
ZmGST 20	GRMZM2G434541	1.93	16.64	10.82	8.91	4.71	8-fold	3
ZmGST 21	GRMZM2G428168	15.61	151.54	198.39	219.32	194.54	8-fold	6
ZmGST 22	GRMZM2G330635	59.24	208.94	140.95	136.98	118.47		6
ZmGST 23	GRMZM2G416632	5.56	211.88	200.78	209.11	143.91	8-fold	3
ZmGST 24	GRMZM2G032856	0.06	9.28	4.88	3.05	1.12	8-fold	1
ZmGST 25	GRMZM2G161905	0.91	51.28	15.15	6.36	1.75	8-fold	3
ZmGST 26	GRMZM2G363540	0.59	0.07	0	0	0	8-fold	
ZmGST 27	GRMZM2G077206	0.08	0.15	0.09	0.06	0		
ZmGST 28	GRMZM2G146475	7.88	16.79	13.13	15.44	11.86		6
ZmGST 29	GRMZM2G127789	0.45	0.70	0.59	0.61	0.44		2
ZmGST 30	GRMZM2G044383	41.73	49.57	30.16	15.25	3.37	8-fold	6
ZmGST 31	GRMZM2G475059	10.05	52.94	40.30	31.53	21.71		6
ZmGST 32	GRMZM2G041685	0	0.38	0.07	0.31	0.45		

ZmGST 33	GRMZM2G028821	0	0	1.28	6.88	11.82		
ZmGST 34	GRMZM2G145069	0	0	0	0	0		
ZmGST 34	GRMZM2G149182	0	0	0	0	0		
ZmGST 35	GRMZM2G161891	0	0.07	0.46	0.87	2.06		
ZmGST 37	GRMZM2G178079	4.09	15.62	17.86	18.05	20.88		6
ZmGST 38	GRMZM2G066369	0.21	0	0.26	0.54	0.29		
ZmGST 40	GRMZM2G054653	0.08	0.37	0.52	0.40	0.68	8-fold	2
ZmGST 41	GRMZM2G097989	26.40	26.00	22.70	32.69	34.13		6
ZmGST 42	GRMZM2G025190	2.10	197.29	133.85	90.71	66.98	8-fold	3
ZmGLP2-1	GRMZM2G045809	1.44	25.16	373.23	412.11	251.27	8-fold	3
ZmGLP3-1	AC190772.4_FG011	0	0.11	7.97	4.56	2.48		
ZmGLP3-2	GRMZM2G030772	0	0	6.04	5.85	1.73		
ZmGLP3-3	GRMZM2G149714	0	0.07	2.77	2.45	1.23		
ZmGLP3-16	GRMZM2G072965	0	0.15	0.24	0.49	0.17		
ZmGLP10-1	GRMZM2G178817	0	0.62	1.49	0.45	0.18		
ZmGLP10-2	GRMZM2G071390	0	0.64	0.59	0.34	0		
ZmGLP10-3	GRMZM2G049930	0	1.39	2.88	0.79	0.32		

[a.]Fragments per kilobase of exon per million fragments mapped (FPKM) at 0, 24, 36, 48, and 72h after placement on tissue culture initiation medium, genes with an 8-fold change in expression or greater as compared to the 0h time point, and the assigned gene cluster designated by k-means analysis.

Table 2.4. Expression of WUSCHEL-related maize WOX genes in tissue culture.[a]

			FPKM						
Gene name	Annotated name	Sequence similarity (%)	0 h	24 h	36 h	48 h	72 h	Fold change	Cluster number
ZmWOX2A	GRMZM2G108933	100	0.70	0.34	0.08	0.04	0.11	8-fold	2
ZmWOX2B	GRMZM2G339751	100	0.18	0.68	0.15	0	0		
ZmWOX3A	GRMZM2G122537	100	0.23	0.98	0.32	0.53	0.39		2
ZmWOX3B	GRMZM2G069028	85.71	1.50	1.10	0.52	0.22	0.79		5
ZmWOX3B	GRMZM2G140083	84.81	0	0	0	0	0		
ZmWOX5A	GRMZM2G478396	96.72	0	4.51	9.06	6.20	6.61	8-fold	
ZmWOX5B	GRMZM2G116063	100	0.06	2.17	1.91	4.53	6.27	8-fold	1
ZmWOX9A	GRMZM2G133972	100	0.49	0.55	0.19	0.07	0		
ZmWOX9B	GRMZM2G031882	100	5.95	3.07	2.91	2.08	1.57		6
ZmWOX9C	GRMZM2G409881	100	5.53	3.01	4.86	3.42	2.85		6
ZmWOX11/12B	GRMZM2G314064	98.46	2.51	11.29	15.50	17.96	14.80	8-fold	3
ZmWOX13A	GRMZM2G038252	100	0	0	0	0	0		
ZmWOX13A	GRMZM2G069274	100	0	0	0	0	0		
ZmWOX13B	GRMZM5G805026	100	5.86	5.46	6.21	7.46	6.05		6
KNOTTED1	GRMZM2G017087	100	20.67	15.33	11.52	10.66	10.94		6

[a.]Fragments per kilobase of exon per million fragments mapped (FPKM) at 0, 24, 36, 48, and 72 h after placement on tissue culture initiation medium, genes with an 8-fold change in expression or greater as compared to the 0 h time point, and the assigned gene cluster designated by k-means analysis.

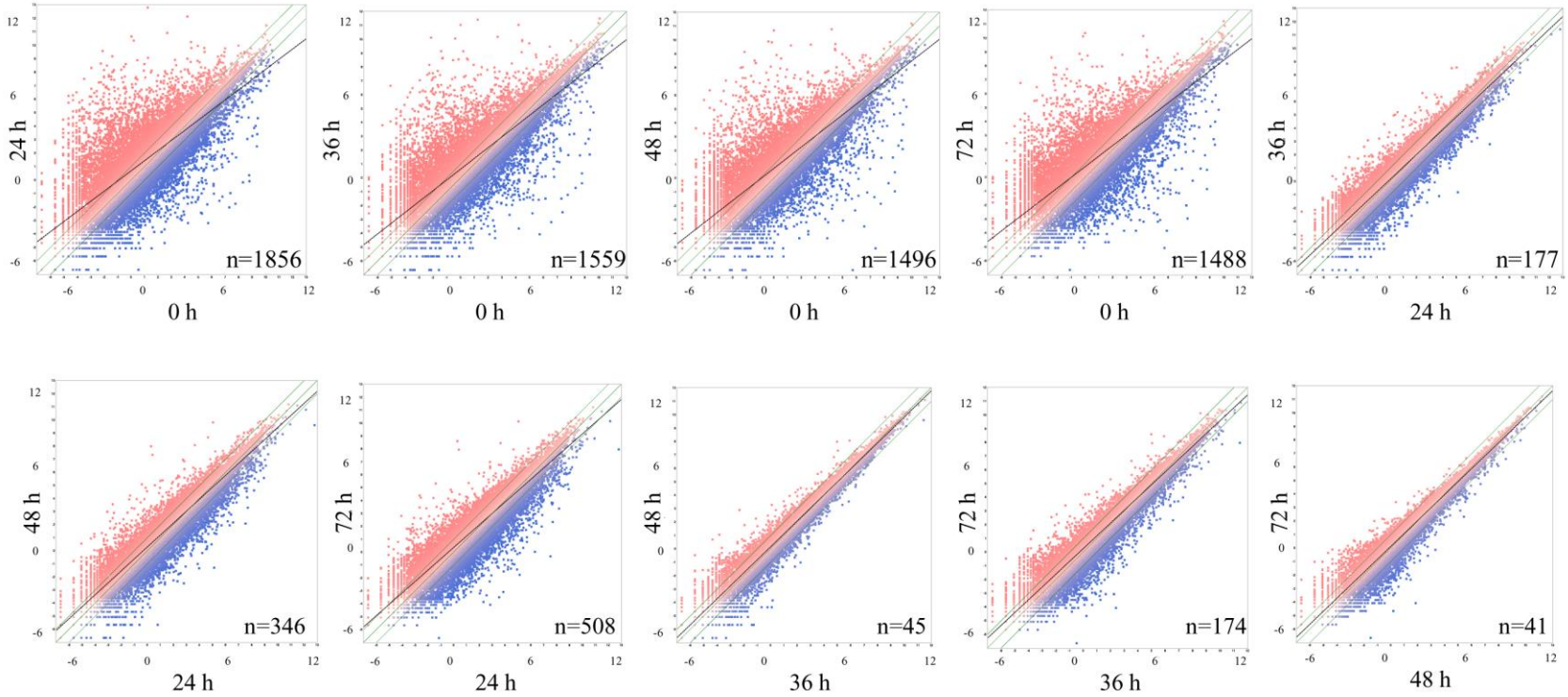


Figure 2.1. Gene expression changes in early somatic embryogenesis. Scatter plots of gene expression changes as log2 values of fragments per kilobase of exon model per million fragments mapped (FPKM) in immature zygotic embryo explants of maize inbred line A188 after placement on culture initiation medium for each time point comparison at 0, 24, 36, 48, and 72 h where n is the number of genes differentially expressed greater than 8-fold for each time point comparison. Red dots represent genes that are up-regulated, blue dots represent genes that are down-regulated, the middle green line indicates no fold change in expression, the two outer green lines indicate a 2-fold change in expression, and the solid black line is the best fit linear correlation

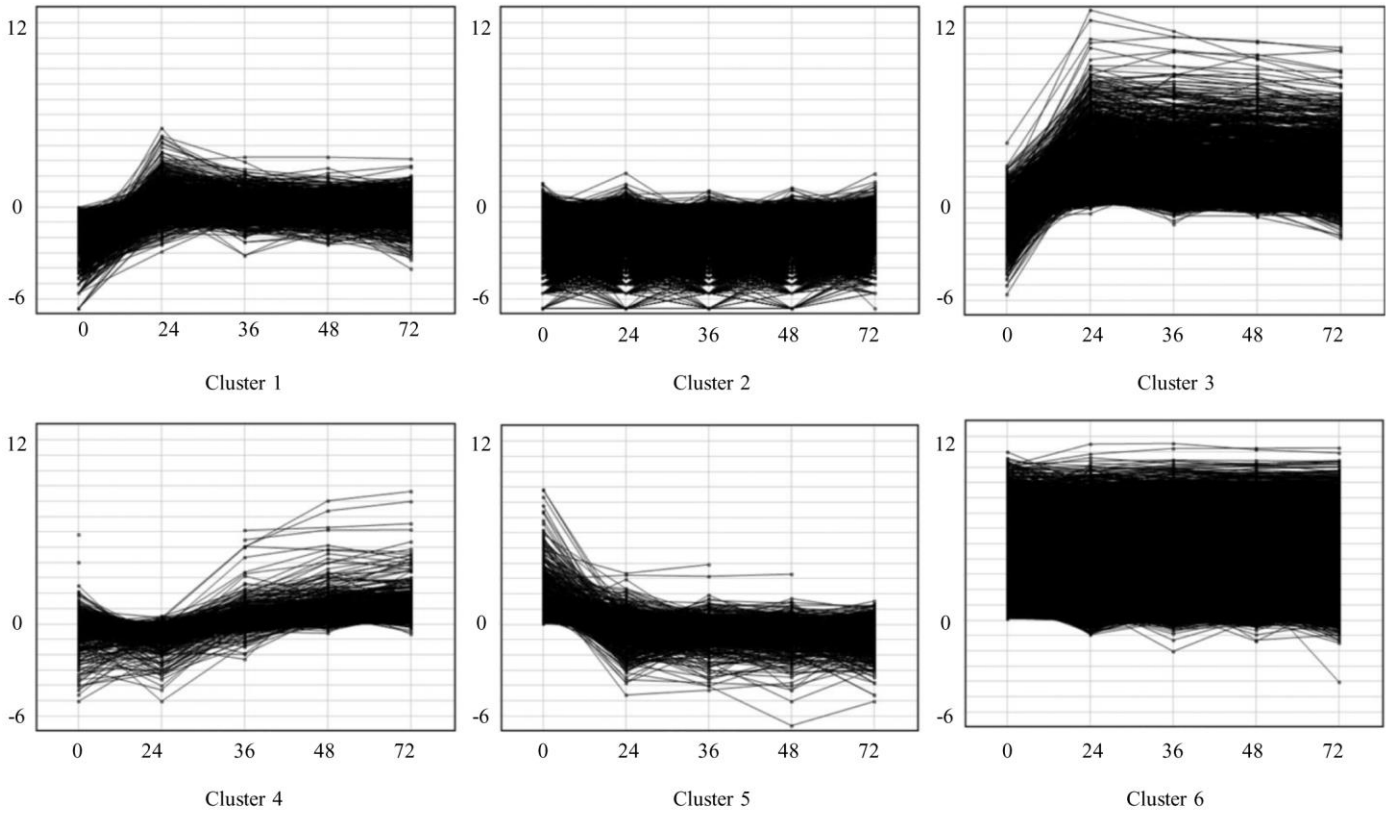


Figure 2.2. K-means clustering of genes expressed during early somatic embryogenesis. Log2 values of fragments per kilobase of exon model per million fragments mapped (FPKM) in genes with greater than zero FPKM expressed in immature zygotic embryo explants of maize inbred line A188 at 0, 24, 36, 48, and 72 h after placement on culture initiation medium grouped by expression trends as uncentered Pearson's correlation coefficient in six k-means clusters

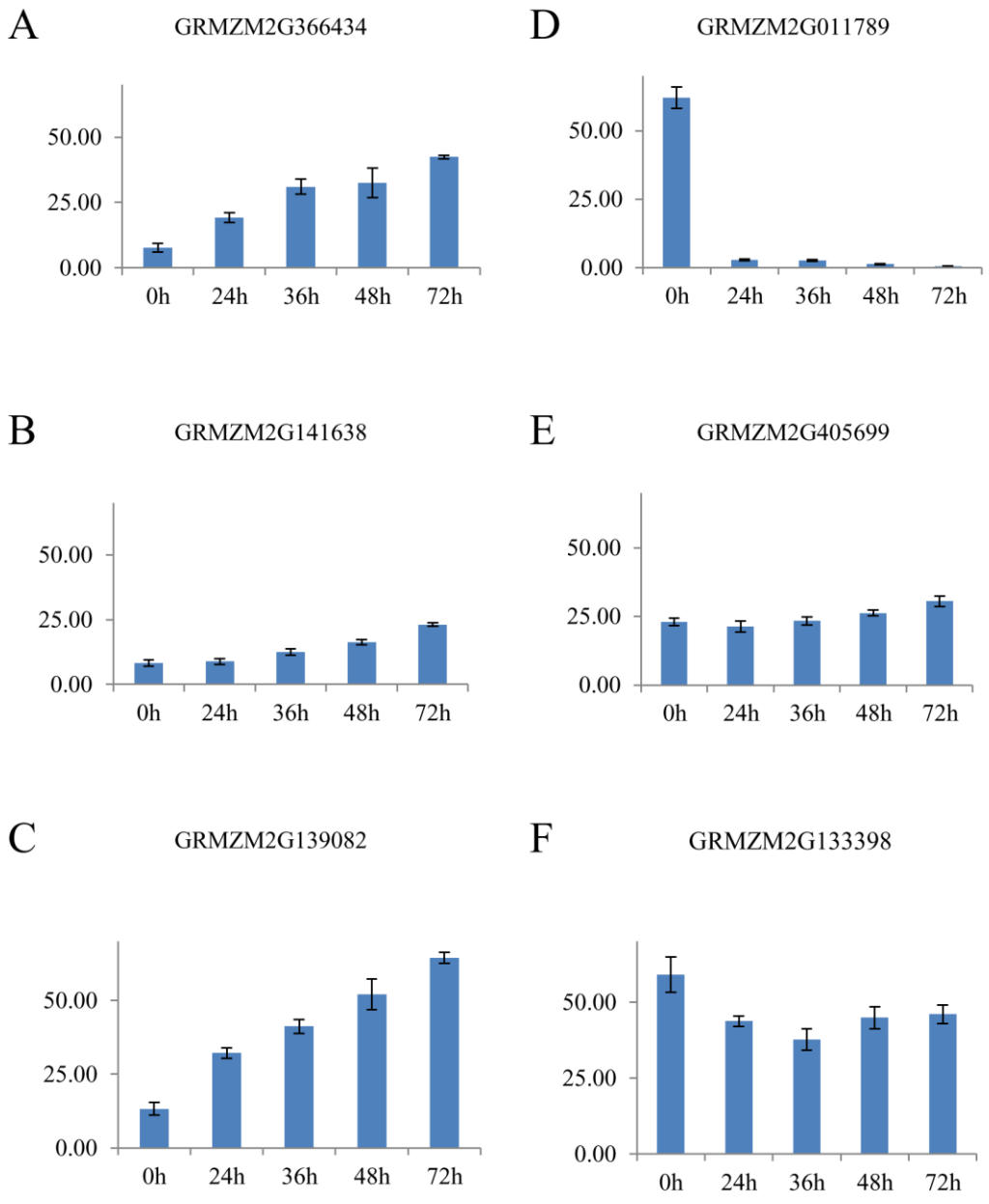


Figure 2.3. Gene expression of somatic embryogenesis genes involved in induction. Average values of fragments per kilobase of exon model per million fragments mapped (FPKM) of genes involved in the induction of somatic embryogenesis expressed in immature zygotic embryo explants of maize inbred line A188 at 0, 24, 36, 48, and 72 h after placement on culture initiation medium where (A), (B), and (C) are BBM-like maize genes with high sequence similarity to *Brassica napus* BABY BOOM (BnBBM1), (D) is maize LEAFY COTYLEDON1 (ZmLEC1), (E) is a maize gene with high sequence similarity to *Arabidopsis thaliana* LEAFY COTYLEDON2 (AtLEC2), and (F) is maize VIVIPARIOUS1 (VP1). Bars indicate average mean ± SE (n=4 for 0, 24, 36, and 48 h include technical and biological replicates; n=2 for 72 h include only technical replicates)

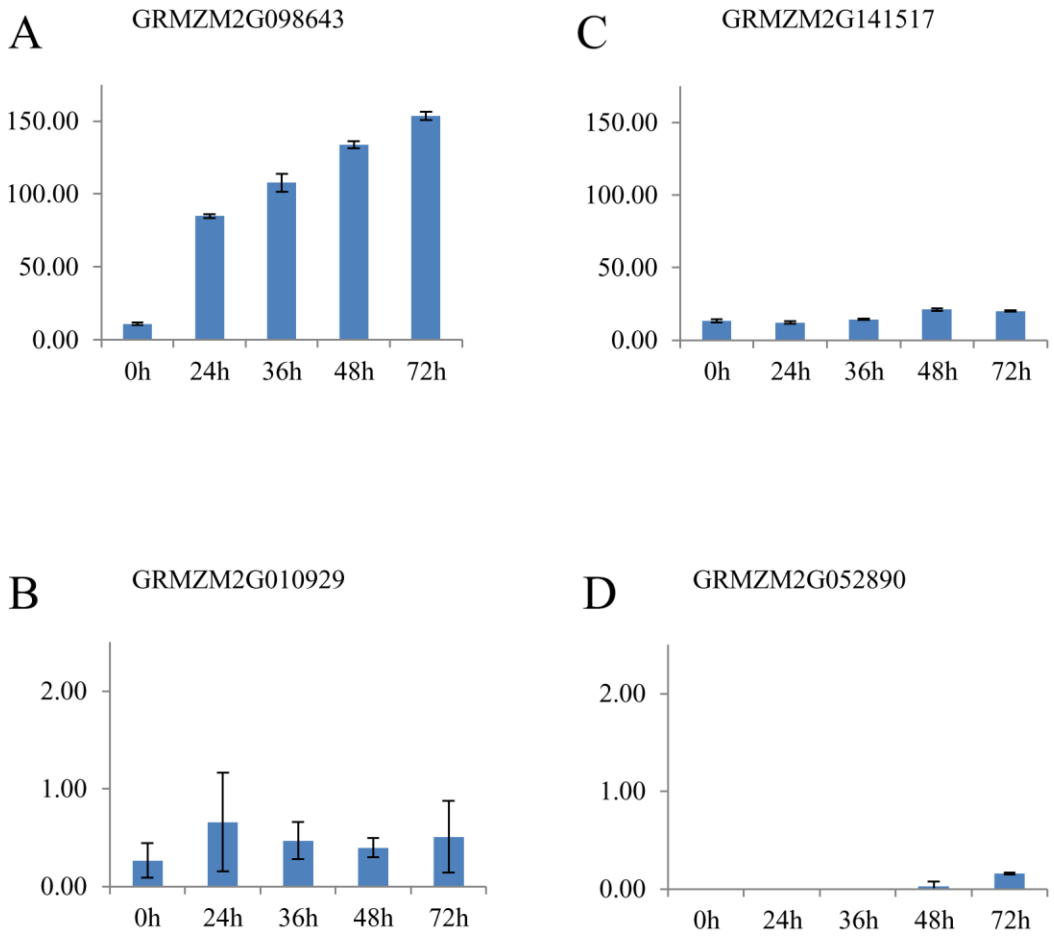
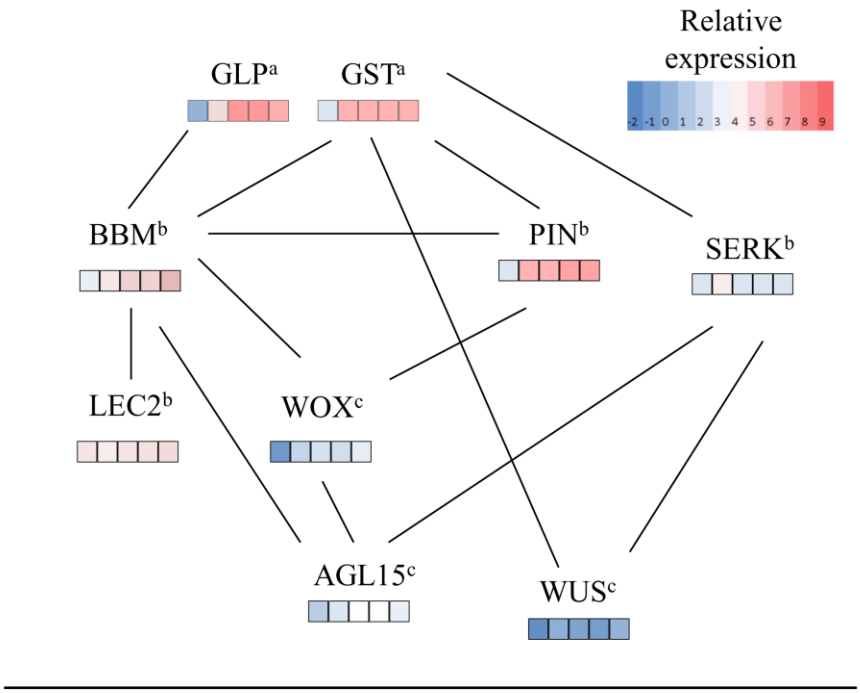


Figure 2.4. Gene expression of somatic embryogenesis genes involved in callus initiation and maintenance. Fragments per kilobase of exon model per million fragments mapped (FPKM) of maize genes associated with embryogenic callus induction expressed in immature zygotic embryos of maize inbred line A188 at 0, 24, 36, 48, and 72 h after placement on culture initiation medium where (A) is the maize ortholog to PINFORMED1 (ZmPIN1a), (B) is the maize ortholog to WUSCHEL (ZmWUS1), (C) is maize gene with high sequence similarity to CLAVATA (CLV1) and (D) is a maize ortholog to AGAMOUS (ZAG1). Bars indicate average mean ± SE (n=4 for 0, 24, 36, and 48 h include technical and biological replicates; n=2 for 72 h include only technical replicates)



[a]Genes most commonly associated with stress response.
[b]Genes most commonly described as being involved in embryogenic pathway initiation.
[c]Genes most commonly described as being involved in somatic embryo formation and development.

Figure 2.5. Relative expression of coexpressed somatic embryogenesis-related genes. A proposed model of somatic embryogenesis-related gene expression networks as determined by coexpression (solid lines) with a correlation coefficient greater or equal to 0.9 between genes expressed during the early stages of somatic embryogenesis. Relative expression of transcripts detected in immature zygotic embryo explant tissues in tissue culture were detected in the inbred line A188 and reported as the average log2 expression displayed by color coded values as depicted by the figure legend for each gene. The first left most box under each gene name is the average log2 transformation of fragments per kilobase of exon model per million fragments mapped (FPKM) at 0 h, the second box at 24 h, the third at 36 h, the fourth at 48 h, and the fifth right most box is the average FPKM 72 h. Glutathione-S-transferases (GSTs) and germin-like proteins (GLPs) are stress response genes that are triggered in early somatic embryogenesis. BABY BOOM (BBM), an APETALA-like ethylene-responsive element transcription factor, and LEAFY COTYLEDON2 (LEC2), a B3 domain transcription factor, promote somatic embryogenesis. PINFORMED (PIN) genes mediate auxin transport and establish essential endogenous auxin concentrations in the cell. SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASES (SERK) genes are also involved in somatic embryogenesis and hormone metabolism. WUSCHEL (WUS), a homeodomain transcription factor, regulates stem cell fate during embryo formation and development. AGAMOUS like-15 (AGL15), a MADS box transcription factor, also promotes somatic embryo formation and is also involved in meristem development. WUSCHEL-related homeobox domain (WOX) genes have also been detected during somatic embryogenesis in embryogenic genotypes but not in non-embryogenic genotypes.

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CHAPTER 3: VALIDATION OF QUANTITATIVE TRAIT LOCI ASSOCIATED WITH REGENERATION ABILITY IN TISSUE CULTURE AND NEAR-ISOGENIC LINE DEVELOPMENT USING MARKER-ASSISTED BREEDING

3.1 INTRODUCTION

It has been nearly a decade since the prestigious journal, Science, published a special issue on "What we don't know," where 125 of some of the most intriguing research questions were revealed that remained to be answered by the scientific community. One of those questions regarded how somatic cells form into whole plants [1]. The answer to this research question remains elusive. The genes and specific pathways involved in the initiation of somatic embryogenesis and leading to plant regeneration ability in tissue culture are still not very well understood. Embryogenic, regenerable tissue cultures are key components of many plant clonal propagation systems and most genetic engineering systems for major crops. Because genotype-dependent embryogenic culture response limits utilization of those applications, deciphering the genetic mechanisms controlling somatic embryogenesis would aid in the development of more responsive germplasm and/or development of genotype-independent tissue culture systems. Although studies investigating the genetic factors underlying embryogenic culture response have been conducted, few have led to the characterization of genes that may play a role in embryogenic, regenerable tissue culture response, and to date, not a single study has pointed to causal genes in major crop plants, such as maize.

One approach toward genetic characterization of embryogenic, regenerable culture response in maize and other crops has been to identify and map quantitative trait loci (QTL) associated with the trait. Identification and analysis of candidate genes within QTLs would

provide important information in gaining a deeper understanding of the biological mechanisms that may be involved in tissue culture response that leads to regeneration ability.

In maize, embryogenic callus formation and morphology are distinguishable and are described as either Type I or Type II calli. Type I calli are typically less efficient in regeneration ability. The more preferred calli type are Type II calli, which are light yellow to white in color, grow relatively fast, and are highly friable and embryogenic and easy to subculture [2]. The first report of Type II regeneration ability in maize was described in 1975 [3] on the maize inbred line, A188. A188 has been utilized in many studies aimed at understanding and improving embryogenic efficiency and regeneration ability of immature embryo-derived Type II callus in maize [4-13]. The maize inbred line, A188, produces highly embryogenic cultures, as well as the Hi II hybrid also characterized as Type II in callus morphology. The Hi II hybrid was developed from a cross between two lines designated as Parent A and Parent B which were selected from an A188 X B73 population [14]. Again, A188 was highly efficient in tissue culture response while B73 had very low tissue culture response. A188 was also characterized by poor agronomics. It was developed from the University of Minnesota [15] and has been categorized in the Iowa Stiff Stalk Synthetic maize heterotic group [16]. It is short in plant height with white kernels. B73 is the recently sequenced maize reference inbred line [17]. It is widely adapted and also an Iowa Stiff Stalk Synthetic. B73 has yellow kernels, good agronomic qualities, but again, is very poor in tissue culture response [14]. The maize inbred line H99, a Reid Yellow Dent, which is also efficient in embryogenic capacity, is characterized with Type I callus morphology, and maize germplasm in China, such as 18-599 and Huangzao4, have been reported to be efficient in Type II tissue culture response [18,19].

Attempts to map QTLs in maize that are associated with tissue culture response have, to date, been done in using embryogenic cultures initiated from immature zygotic embryos [7,15,18-20] (Table 3.1). These studies differ, however, in types of molecular markers used, from restriction fragment length polymorphisms (RFLPs) to simple sequence repeats (SSRs) to single nucleotide polymorphisms (SNPs). In addition, these QTL studies in maize tissue culture used different genotypes with high embryogenic capacity such as A188, Hi II, and H99 for example. Other difference between these QTL studies are the mapping strategy used and methodology for population development. Some studies used multiple regression, segregation distortion and composite interval mapping to detect significant loci. Similarly, some studies involved backcross-derived mapping populations, recombinant inbred line (RIL) populations and segregating populations such as F_2 and $F_{2:3:4}$ lines. Regardless of the many differences in experimental design in these QTL studies on maize tissue culture, some of the QTLs reported among these studies were physically located on the same chromosomal arms. In a QTL study on tissue culture traits using the A188 X B73 bi-parental mapping population, QTL regions associated with tissue culture response [7] were similar to another study also involving QTLs identified in a backcross-derived mapping population between a non-regenerable maize inbred line FBLL, also a stiff stalk line, and the highly regenerable Hi II hybrid [15]. Specifically, the QTL regions in common were on the short and long arms of chromosome 1, the long arm of chromosome 2, and a segment on the long arm of chromosome 3. Moreover, three separate and distinct maize QTL studies also reported QTLs on chromosome 3 attributed to genetic contributions from inbred lines A188, H99, and Chinese maize germplasm 18-599 and Huangzao4 [7,15,18-20].

QTL mapping has been successful in facilitating marker-assisted breeding efforts in plants [21]. However, when the objective is to identify candidate genes that are responsible for the trait of interest, validating QTLs requires additional analysis and population development in order to gain higher resolution mapping throughout the genome that enables positional cloning the genes of interest [22]. QTL detection and estimation in near isogenic lines (NILs) developed by backcrossing are advantageous for verifying QTLs because the genome is more homozygous and the effect of background genetic variation is minimized [23].

In this study, the overall goal was to validate embryogenic, regenerable culture response-associated QTLs detected in a backcross derived mapping population between the highly embryogenic maize inbred line, A188, and the maize reference genome inbred line, B73. Putative QTLs were previously detected in 134 BC_3S_3 lines by screening for tissue culture response and genotyping with 89 SSR markers. Significant QTLs were reported between chromosomal bins 3.05 and 3.07 and between chromosomal bins 7.03 and 7.05. Seven BC_3S_3 lines displayed high embryogenic capacity as defined by exhibiting a high number of embryogenic structures per immature zygotic embryo. One line displaying very high embryogenic capacity, designated as ARC60, was selected from this BC_3S_3 population for further mapping and QTL validation. Additional backcrossing and selfing with ARC60 to the recurrent parent, B73, combined with phenotypic screening and additional SNP marker analysis revealed significant QTLs associated with embryogenic response on chromosomes 2, 3, and 4. The specific objective of this study were is to validate QTLs having significant effects on embryogenic culture response by (1) testing the effect of the putative QTLs on chromosomes 2, 3 and 4, (2) further screen the population using marker-assisted selection to obtain near-isogenic lines, and (3) testing the effect of the putative QTL on chromosome 3 using near isogenic lines

with different introgressions. The research was conducted to advance toward the long-term goal of identifying candidate genes affecting embryogenic capacity and regeneration ability in maize. Identification and characterization of the genes will enhance understanding of the genetic mechanisms underlying somatic embryogenesis, and may aid in the development of germplasm with enhanced culture response or the design of genotype-independent tissue culture systems

3.2 MATERIALS AND METHODS

Population development

Select BC_2F_1 ARC60 families were grown in the field in 2010 (n=250) and also in the greenhouse in 2010 (n=800) for screening and marker-assisted selection. In 2011, BC_2F_2 and BC_3F_2 families (n=240) were also grown in the field and greenhouse, again to select individual plants for developing near-isogenic lines. In 2012, $BC_2F_{2:3}$ (n=384) were grown in the greenhouse for selection of homozygous lines. In addition, a random set of BC_2S_1 ARC60 families were sent for doubled haploid induction in the spring of 2010. DNA was extracted from young seedlings or green leaf tissue samples at the V1 to V2 plant stage using a 96-well plate format quickprep extraction method (Appendix A.1) or a modified CTAB extraction method for high quality DNA (Appendix A.2).

Genotyping

In order to observe genomic regions of introgression of A188 in the ARC60 germplasm, ARC60 was genotyped using the 1536-SNP (Illumina, San Diego, CA) , an Illumina GoldenGate genome-wide marker assay designed for maize. Polymorphic markers were selected for marker-assisted breeding to identify plants with introgression on or near the putative QTLs on chromosomes 2, 3, and 4. Additional introgressions were also identified in ARC60 that were not

previously known on chromosomes 1, 7, 9, and 10. In 2012, the University of Wisconsin Biotechnology Center (Madison, W) prepared the MaizeSNP50 BeadChip (Illumina, San Diego, CA), with 55,000 evenly space markers providing genome wide coverage, to genotype ARC-60 again (Figure 3.1) in addition to four backcross-derived near-isogenic lines (NILs), and a bulked sample of ARC60 progeny families that represented the seeds sent for doubled haploid induction. Using this high resolution genotyping information, polymorphic markers were selected for Sequenom (San Diego, CA), a MALDI-TOF mass spectrometry genotyping assay provided by services from the Genomic Technologies Facility at Iowa State University (Ames, IA) for further NIL development. Using Sequenom to increase the resolution for the region on chromosome 3, 31 SNP markers were selected to screen the doubled haploid lines to identify NILs for study.

Greenhouse growing environment

Plants for experiments to test the effect of putative QTLs were grown in the greenhouse at the University of Wisconsin Walnut Street Greenhouse Complex (Madison, WI). Single plants were grown to maturity in classic 2000 pots (19:3 L; Hummert International, Earth City, MO). Pots were prepared with Scotts Metro-Mix 360 soil (The Scotts Company, Marysville, OH) and 32 g of Osmocote Plus 19-5-8 controlled-release fertilizer (Hummert International, Earth City, MO). For seedling emergence, seeds were placed in pots full of Metro-Mix and drenched with water. After sowing single seeds one inch from the soil surface, pots were watered lightly to cover the seed with soil and to ensure ample soil compaction. After sowing, the pots were left without watering again for approximately one week. After emergence, plants were watered every two days to every other day until after the fourth-leaf stage of plant development. At the V8 stage of physiological maize development, when the 6^{th} leave collar is showing, based on the Federal Crop Insurance Corp for staging corn, the pots were treated with liquid fertilizer twice a

week. Greenhouse fertilizer mixed with water delivered 350 ppm of nitrogen and contained 20-10-20 Peters Professional (The Scotts Company, Marysville, OH) water soluble fertilizer with elevated levels and chelated micronutrients. Plants were grown under natural light and supplemental lighting conditions year round (16 hr. light: 8 hr. dark). The average light level one meter from the greenhouse floor was 580 µmoles from artificial light provided by 1000 watt high pressure sodium bulbs. Temperatures were maintained at 28ºC. Ear shoots were covered after emergence and controlled pollinations were made to ensure self-pollinating of each plant entry. Four weeks after pollinations, husks were pulled back and allowed to dry and watering ceased. Plants were left to dry out for an additional 3 weeks until harvest.

Harvest

Ears of field grown plants were harvested 40 days after pollination and then stored in the 90 degrees Fahrenheit grain dryers at West Madison Research Station for 7 days. Similarly, ears of greenhouse grown plants were harvested after pollination and dry down period, and then placed on a blower apparatus at room temperature at the University of Wisconsin Seeds Building for 7 days before shelling. Seed from single ears were harvested by hand into a single ear packet and kept in a cold storage at 55 to 60 degrees Fahrenheit under controlled humidity (55%).

Tissue culture

In order to assess tissue culture response in this study, immature zygotic embryos were harvested from greenhouse grown plants and used as explants to initiate tissue culture growth. Approximately 12 days post pollination when immature embryos were 1.2-1.5 mm along the longitudinal axis from end to end, half of the ear was harvested. The remaining cob was left to develop into mature seeds for the next generation. The harvested portion of the ear was prepared

for tissue culture by sterilizing for 20 minutes in 50% bleach and sterilized water solution containing a drop of Tween-20 followed by three washes with sterile water. All work was conducted in a sterile, laminar flow hood. A total of 50 immature embryos per ear was isolated and placed scutellum side up (embryo axis side down) onto two plates (25 embryos per plate) containing initiation/maintenance medium with the following formulation autoclaved for sterilization for 30 minutes and allowed to cool before pouring onto plastic 100 mm x 25 mm 2 inch petri plates: N6 salts [24] and 1000 X vitamins [1g/L thiamine-HCL, 0.5g/L pyridoxine-HCL, 0.5g/L nicotinic acid, and 2g/L glycine], 2.0 mg/L 2,4-D, 2.875g L-proline, 30g sucrose, 8.5 mg/mL or 50 µM $AgNO_3$ (Appendix B). Plates were wrapped with microspore tape and placed in a dark growth chamber for 10 days in the dark at 28ºC. At 10 days, the growing embryonic axis was excised and the embryos were transferred onto fresh initiation medium. Callus was transferred onto fresh media every two weeks for a total of 38 days on initiation medium where, after the second subculture, only the 10 highest responding embryos based on callus diameter and embryogenic mass were selected for continued subculture. The embryogenic tissue was then transferred to Regeneration Medium 1 (RM1) consisting of 4.3 g/L Murashagie and Skoog salts [25], 60 g/L sucrose, 0.1 g/L myo-inositol, 1 mL/L 1000X MS vitamin stock [0.5 g/L thiamine-HCL, 0.5 g/L pyridoxine-HCL, 0.05 g/L nicotinic acid, and 2 g/L glycine] (Appendix B). RM1 was prepared by autoclaving for 30 minutes and allowed to cool to 65-70 degrees C. Filter sterilized stock solutions were then added to the RM1 including 1 mg/L IAA, 0.5 mg/L zeatin, and 0.023 mg/L ABA, and the medium was poured into sterile, 100mm x 25mm plastic petri plates. RM1 plates containing the transferred tissues were kept in an incubator in the dark at 28ºC for 10-14 days. The tissue was then transferred onto Regeneration Medium 2 (RM2) consisting of 4.3 g/L MS salts, 40 g/L sucrose, 0.1 g/L myo-inositol, 1 mL/L 1000X MS vitamin

stock [0.5 g/L thiamine-HCL, 0.5 g/L pyridoxine-HCL, 0.05 g/L nicotinic acid, and 2 g/L glycine], and hormone stock solutions at 1mg/mL (1 mL/L IAA, 0.5 mL/L zeatin, 23.2 µL/mL ABA) (Appendix B). Plates containing transferred tissues were stored in an incubator at 26ºC with 16 hr. light: 8 hr. dark photoperiod to allow for shoot formation. Light intensity (at 100-150 µmol $m^{-2}s^{-1}$) was supplied by 17 watt Phillips F17T8 bulbs set at 16 cm above the plates. All media was pH 5.8 and solidified with 3.5g/L Gelzan. Tissue was kept on RM2 for 14 days.

Experimental design for Experiment I: A preliminary validation test for putative QTLs associated with regeneration ability - digenic versus monogenic NILs

A preliminary test to validate culture response-associated QTLs in an ARC60 X B73 backcross-derived mapping population included an experiment to compare the effect of monogenic NILs containing a single putative QTL on chromosome 2 to digenic NILs containing two QTLs, one each on chromosomes 3 and 4. In 2011, four experimental BC_2S_2 lines: MAB52, MAB54, MAB84, and MAB94 were tested for regeneration ability representing the two different genotypic classes. Lines MAB52 and MAB54 harbored a single A188 introgression at the putative QTL on chromosomal bin 2.08. MAB84 and MAB94 harbored two QTLs, one large A188 introgression on chromosomal bin 3.06 and 3.07 and one small A188 introgression on chromosomal bin 4.03. Eight reps per genotype were tested for the effect of the QTL on embryogenic, regenerable culture response, and each rep included A188, ARC60, and the HiII hybrid as positive controls and the maize inbred line B73 as a negative control. Single plants were placed in the greenhouse in a completely randomized design. Fifty immature zygotic embryos were isolated from each ear (per plant, genotype and rep) and placed onto tissue culture initiation medium. The 10 highest responding embryos based on callus diameter and embryogenic mass were selected for each ear and then, the number of plantlets regenerated per

ear as the sum of the plantlets regenerated cumulatively across all 10 selected zygotic embryos was counted 10-14 days after placement on RM2 to assess plant regeneration ability as tissue culture response.

Experimental design for Experiment II: A validation test for a putative major QTL associated with embryogenic regeneration ability on chromosome 3 using near-isogenic lines

In order to test the effect of a putative QTL on chromosome 3 associated with regeneration ability, NIL genotypes with varying size and physical location of A188 introgressions spanning chromosomal bin 3.06 and 3.07 were included in a greenhouse study. NIL genotypes DH78, DH99, DH240, MAB417, MA485, MAB84 and MAB94, along with positive and negative control plants B73, A188, ARC-60 and HiII, were grown in two separate greenhouse locations in a completely randomized design with multiple planting dates per greenhouse location. Each growing environment (greenhouse and planting time) had three replicates or three plants per genotype in each planting. The first planting time was October 2, 2012 in both greenhouses. The second planting time was October 23rd in both greenhouses. A third planting time on November 13th, 2012 was planted in only one greenhouse due to low seed quantities. Similarly, fifty immature zygotic embryos from each ear were isolated for tissue culture. The number of zygotic embryos producing embryogenic callus was counted after 38 days on maintenance media. Twenty total embryos were selected for regeneration testing and the number of plantlets generated per selected embryo was counted at 7-14 weeks after placement on regeneration 2 media.

Data Analysis

Data analysis was conducted using SAS v9.3 (SAS Institute, Cary, NC) (Appendix C.1). For the analysis of putative QTLs using updated SNP marker information, marker-trait associations were assessed by performing single marker-trait analysis using the proc glm in SAS to detect significant differences between the mean values of A188 and B73 inbred maize lines at each marker loci. The phenotypic variation among the entry means, R^2, was accounted for by each marker allele.

For Experiment I, the test to compare monogenic versus digenic NILs, a non-parametric test in SAS using the npar1way function was used to determine the significant differences between mean distributions in lines tested with a Kruskal-Wallis test (H_o: $\mu_i=\mu_j$), where the null hypothesis was that there are no differences between genotypic means in terms of the average number of plantlets generated per plant. A significant result in the Chi-square test allowed for the conclusion that there were significant differences between genotypic means. The test for normality using the Shapiro-Wilk test was done in SAS using proc univariate to obtain a histogram and a measure for skewness in the data set. Finally, a t-test was used to compare groups of genotypes such as digenic NILs MAB84 and MAB94 versus monogenic NIL types MAB52 and MAB54. The t-test determined the significance equality of variances between the two groups and a Chi-square test was used to determine the significance of differences between the distributions of means.

Similar data analysis was done for Experiment II including the non-parametric one way test for differences in means and the test for normality. In addition, a test for the robustness of the experimental design was done using an analysis of variance (ANOVA) using proc glm to determine the significant sources of variation. Briefly, the following linear models were used:

Model I: $Y \sim \mu + G + T + L + R(L) + TxL + GxT + GxL + GxTxL + \varepsilon$

Model II: $Y \sim \mu + G + T + L + R(T) + GxT + \varepsilon$

Where Y is the observed trait measured as the number of plantlets regenerated per plant as a sum from the 20 best selected zygotic embryos for each plant, to determine regeneration ability or tissue culture response. μ is the overall mean, G are genotypes, T is planting time, L is greenhouse location, R is replicates which are nested within greenhouse location. In addition, the following interactions were accounted for in Model I: TxL is interaction between planting time and greenhouse location, GxT is the interaction between genotype and planting time, GxL is the interaction between genotype and greenhouse, and GxTxL is the interaction between genotype, planting time, and greenhouse location. Since only the GxT interaction was significant in Model I, Model II was used to account for only the GxT interaction. All other interactions in Model I were dropped from the analysis because they were not significant. Model II accounts for G, T, and L similar to Model I, however R(T) is replicates within planting time was included and only the interaction between genotype and plating time considered because all other sources of variation were non-significant. Genotypes, planting time, and location were considered as fixed effects and replicates were considered as random effects. A least squares mean was determined for each genotype and the standard error calculated using lsmeans statement in SAS.

3.3 RESULTS

Identification of putative quantitative trait loci associated with embryogenic, regenerable culture response in a BC_2S_1 backcross derived mapping population

In order to gain more information on genotypes with confirmed embryogenic regeneration ability, high resolution genotyping was used to analyze the BC_3S_3 ARC60 line

(Figure 3.1) so that additional polymorphic SNP markers (Table 3.2) could be identified. Lines from a previous study [26], where BC_2S_1 ARC60 plants were phenotyped for embryogenic capacity in tissue culture, were genotyped with the additional SNP markers in this study and single-marker trait analysis using the new SNP data confirmed significant putative QTLs (Table 3.3). This analysis revealed several differences as compared to the previous study. In the previous study, the QTL on chromosome 2.08 was significant only by allelic means; however with the new SNP genotypes and marker-trait associations, a QTL on chromosome 2 was shown to be significant (*P-value* 0.009). In addition, the new analysis did not confirm a QTL on the long arm of chromosome 4 as previously reported, but a QTL on the short arm of chromosome 4 was detected (*P-value* = 0.02). In summary, additional SNP genotyping and subsequent single-marker analysis identified putative QTL on chromosomal bins 2.08, 3.06, 3.07, and 4.03 explaining 12% (*P-value* 0.009), 14% (*P-value* 0.01), 21% (*P-value* < 0.001), and 13% (*P-value* = 0.02) of the phenotypic variation, respectively (Table 3.3).

Marker-assisted breeding: Line development for Experiment I.

A total of 37 polymorphic SNPs (Table 3.2) were utilized for initial marker-assisted breeding to aid in identifying and developing near isogenic lines to test for the effect on embryogenic, regenerable culture response of putative QTLs on chromosomes 2, 3, and 4. In summer 2010, 21 BC_2F_1 ARC60 families were planted in the field where each family was planted in two rows with 15 kernels per row. Single plants (n=209) were genotyped with the 37 polymorphic SNPs before flowering to select plants for selfing and backcrossing. In summer of 2011, BC_2F_2 progeny from summer 2010 were grown in the field. Single plants (n=240) were genotyped with only 8 SNPs to select for informative genotypes harboring only the putative QTLs of interest. Near isogenic lines developed through marker assisted backcross breeding

(Figure 3.2) were identified in 2011 and then genotyped again using 55,000 SNP coverage. Two breeding lines designated as MAB52 and MAB54 were near isogenic to the recurrent parent maize inbred B73 with 99.5% and 99.6 % of the genome identical to B73, except both lines harbored a small genomic segment of maize inbred line A188 on chromosome 2. MAB52 had a region of the putative QTL on chromosome 2 with A188 segments physically located between 209MB to 235MB, and MAB54 had a region of the putative QTL on chromosome 2 spanning 209MB to 231MB (Table 3.4). Two additional breeding lines, designated as MAB84 and MAB94 were also isogenic to the recurrent parent, except both lines harbored two small segments of the maize inbred line A188, spanning the region of the putative QTL on chromosomes 3 and 4 (Table 3.4). MAB84 had A188 segments on chromosome 3 between 164MB to 167MB, and 171MB and 194MB in addition to a segment on chromosome 4 between 10MB to 13MB. MAB94 had A188 segments on chromosome 3 between 164MB to 167MB, 171MB to 195MB, and 203MB to 208MB. In addition, MAB94 also had an A188 segment on chromosome 4 between 10MB and 14MB.

Marker-assisted selection: Line development for Experiment II.

Additional breeding lines were developed using marker-assisted selection focusing on developing NILs maintaining A188 introgressions in only one region on chromosome 3 with the overall objective to test for the effect on embryogenic, regenerable culture response of a putative QTL spanning chromosomal bins 3.06 and 3.07. A BC_3F_2 line designated as MAB417 was identified in summer of 2011 and a $BC_2F_{2:3}$ line designated as MAB485 was identified in the greenhouse in winter 2010 (Figure 3.3).

In addition, 500 kernels from 30 randomly selected BC_2F_1 ARC60 families (Table 3.5) were sent to generate doubled haploids. In order to study fixed or segregating regions in the genome, DNA samples from10 seedlings from each family were pooled and then genotyped with 55,000 SNPs using high density, genome-wide marker coverage. Using the genotyping information on pooled samples, a marker project was created with 30 SNPs (Table 3.6) to determine useful genotypes and extent of the proportion of the genome that was B73 versus A188 introgressions. Seeds that had been induced to produce haploids, doubled, and selfed (n=244) were planted in the greenhouse and genotyped again as single plants using Sequenom. Again, marker-assisted breeding using Sequenom genotyping allowed for selection of doubled haploid (DH) lines with A188 segments on chromosome 3. DH NIL lines were validated by genotyping again as single plants using MaizeSNP50. Three DH lines were maintained for further study. DH78 had one small A188 genomic segment on chromosome 3 between 203MB to 209MB. DH99 had two small segments on chromosome 3 at 164MB to 167MB and another on 178MB to 188MB. DH240 had 3 segments on chromosome 3 between 7MB to 8MB, 164MB to 167MB, and 178MB to 199MB (Table 3.7).

Experiment I. Test to compare NILs with putative quantitative trait loci associated with embryogenic, regenerable tissue culture response: digenic versus monogenic near-isogenic lines

Five single plant entries were studied for each test genotype: test lines MAB52, MAB54, MAB84, and MAB94 and control lines A188, B73, ARC60 and HiII, where a test for normality in the regeneration response data set displayed a skewness of 1.52 (Table 3.8) with a significant test for normality using the Shapiro-Wilk test ($P < 0.0004$) (Table 3.8) indicating a non-normal data set. To determine the significant differences between mean distributions in lines tested, a Kruskal-Wallis test showed significant Chi-square ($P > 0.0088$) (Table 3.9, Figure 3.4) based on

the distribution of Wilcoxon scores. The goal of this experiment was to determine if monogenic versus digenic NILs performed differently. The highest number of plantlets regenerated from 20 embryos in this study came from a single test genotype MAB94, a digenic NIL, with 19 plantlets regenerated. The highest number of plantlets regenerated from a monogenic NIL was MAB52 at 2 plantlets. A t-test grouping the digenic NILs MAB84 and MAB94 and comparing them to the monogenic NIL types MAB52 and MAB54 showed a significant difference between the equality of variances based on an F-test ($P > 0.0074$), however, the t-test for equal variances was not significant ($P > 0.2272$) (Table 3.10).

It is worth noting that when genotyping MAB84 and MAB94 at a later generation using MaizeSNP50, the data revealed a loss of the putative QTL on the short arm of chromosome 4. Therefore, we did not test for the effect of a monogenic NIL harboring a putative QTL on chromosome 4 in this study. Subsequent selfing generations to increase seed resulted in a loss of the genotype, suggesting that the original detection of a small segment on chromosome 4 that was associated with significant QTL response was at the heterozygous state.

Experiment II. Test to compare near-isogenic lines with varying introgressions of putative quantitative trait loci on chromosome 3 associated with embryogenic, regenerable tissue culture response

In order to compare embryogenic, regenerable tissue culture response in NILs harboring different segments of the A188 introgression on chromosome 3, with a long-term goal to identify genes having large effects on tissue culture response, a greenhouse experiment was conducted in two greenhouse locations with two planting times and three replicates for each of the following genotypes: B73, A188, ARC-60 (negative and positive controls) and the following test NILs

with chromosome 3 introgressions, DH78, DH99, DH240, MAB417, MA485, MAB84 and MAB94 (Figure 3.5). This experiment successfully determined that the NIL genotypes tested each with introgressions varying in size and physical location in the genome were indeed different in their capacity to display embryogenic, regenerable response. The overall objective of this experiment was to determine the regeneration ability of each line to select one NIL line to move forward for fine-mapping.

The total number of plantlets regenerated per plant (from 10 of the highest responding immature zygotic embryos plated per entry) was counted to assess regeneration ability. In order to assess the distributional assumptions of using an analysis of variance model (ANOVA), a statistical test was done to determine if the data met the assumption for normality of sampling distribution. In SAS, the proc univariate command was used to generate a histogram and test for normality. Control genotypes B73 and Hi II performed as expected, with the B73 inbred displaying zero regeneration ability and Hi II displaying very high regeneration ability. Control maize line A188, also displayed high regeneration ability over the NIL genotypes. The data displayed a positively skewed distribution with a skewness rating of 2.09 (Table 3.11). The data was not normally distributed based on a significant Shapiro-Wilk test statistic (*P-value* <0.0001) (Table 3.11). An ANOVA was conducted to gain insight on the significant effects and interactions in the experimental design such as effects due to planting time, greenhouse, and repetitions of each plant nested within greenhouse, genotype, and all possible interactions. The ANOVA resulted in significant effects due to genotype, as expected (*P-value* < 0.0001) and a significant source of variation due to time (*P-value* > 0.0021). There was also significant interaction between genotype and planting time (*P-value* < 0.0001) (Table 3.12). All other sources of variation and interactions as determined by the model were non-significant. The

significant interaction between genotype and planting time may be attributed to poor data capture for one greenhouse and planting time in particular where only eight out of 30 plant entries were successfully tested for regeneration ability. Failure to test the additional entries was attributed to poor plant health and therefore the inability to harvest embryos to test in tissue culture. It is worth noting that due to poor plant health and pest management issues during the experiment, only 52 out of the 120 plant entries had been successfully tested in tissue culture. One greenhouse growing environment fewer instances of missing data. In order to generate the best assessment for a mean values and standard error for each genotype tested, an ANOVA was done for only one greenhouse (Table 3.13). In this model, regeneration ability was determined by effects due to planting time, greenhouse, and repetition nested within planting time, genotype, and the time by genotype interaction. In this model, genotype was significant (*P-value* > 0.0005) and the time and genotype interaction was also significant (*P-value* > 0.12). All other sources of variation were non-significant. In order to obtain a least squares mean and standard deviation, two lines: one positive control line, A188, and the digenic NIL line MAB84 were removed from the analysis due to lack of repetitions in each of the two planting times. ARC60 had the highest LSMEANS, as expected (Table 3.13, Figure 3.6). B73 and DH78 had the lowest LSMEANS (Table 3.13). DH78 had a negative LSMEANS of -5.47 (Table 3.13). Although their raw data values indicated zero total plantlets regenerated, the LSMEANS function takes into account the differences between means within groups and is therefore also called an estimated population marginal mean. LSMEANS are negative because of unbalanced sample sizes and zero values [27]. MAB417, DH99, MAB485, DH240, and ARC50 had high regeneration ability response (Table 3.13, Figure 3.6).

Since the objective of this experiment was to determine if the introgression led to regeneration ability, and also since the data was non-normal, a non-parametric test was also conducted. The NPAR1WAY in SAS was used to run a non-parametric, one way analysis to determine if genotypes tested in the experiment were significantly different. Based on a significant Kruskal-Wallis test, testing for equal means (H_0: $u_i = u_j$), the experiment taking into account all data from both greenhouses was significant, (P>0.0001) allowing the conclusion that the NILs tested had different means (Table 3.14). DH99 showed sufficient regeneration ability with a means score of 26.17. In addition, it had the genotype with the smallest introgression on chromosome 3 (Figure 3.5), making this line a good candidate for further fine-mapping.

3.4 DISCUSSION

A major quantitative trait loci associated with embryogenic, regenerable tissue culture response exists on chromosome 3

One of the most important findings in this study was the confirmation that an embryogenic, regenerable tissue culture response-associated QTL, and therefore potential candidate gene or genes, are present on chromosome 3 in this backcross-derived mapping population. Evidence alluding to this finding stem from both of the QTL validation experiments conducted in this study: the preliminary study involving a comparison between digenic and monogenic NILs with putative QTLs associated with tissue culture response, and the final study that tested the effect of the putative QTL on chromosome 3 with isogenic lines with varying sizes of the A188 introgression. The result in the first study indicated that the mean comparisons between digenic and monogenic NILs were not significantly different. This may be due to the lack of replicates and data points to effectively test the differences between the two genotypic

classes. However, the significant Chi-square test statistics allows for the conclusion that the genotypes tested were different in mean values for regeneration ability. Similarly, when we look at the results from the second study comparing NILs with various introgression on chromosome 3 the genotypic means showed significant differences. In this study, we successfully identified a number of NILs with high regeneration ability, some of which were produced using a doubled haploid approach to obtain not only isogenic lines based to high density marker coverage, but also homozygous lines, which is beneficial for phenotypic testing, eliminating effects due to segregation or heterozygosity. Since some of the NILs developed by doubled haploid induction were successful in displaying consistent embryogenic, regenerable tissue culture response, selecting one of those lines would be optimal for a subsequent study in fine-mapping. DH99 was selected for use in further studies since it had high culture response and its genotype had the smallest introgression, which should aid in efficient progress toward candidate gene discovery and allow for focused high density marker coverage in a relatively small region in question, which is beneficial for a map-based approach.

Minor effect quantitative trait loci associated with tissue culture response on chromosomes 2 and 4 remain inconclusive

In an attempt to test the effect of a monogenic NIL with a chromosome 2 introgression, genotypes MAB52 and MAB54, representing a putative QTL on chromosome 2, did not display high regeneration ability. However, it is important to note that Experiment I had few successfully tested replicates due to novice tissue culture propagation. In addition, this study was unable to effectively test the effect of a chromosome 4 isogenic line since the genotype was lost in subsequent selfing generations, suggesting that the initial detection of the QTL was based on genotypes in a heterozygous state. The observation that the ARC60 line consistently displays

high regeneration ability over the test NIL genotypes included in this study suggests that there could be additional QTLs that enable optimal tissue culture response. A potential forward approach to uncovering the genetic mechanisms that confer embryogenic, regenerable tissue culture response in maize would be to further validate the epistatic interactions between these putative QTLs by conducting a study that tests the effect in isogenic lines in all possible combinations of the A188 introgressions on chromosomes 2, 3, and 4. Additional population development involving successfully isolating a chromosome 4 QTL, and then conducting crossing pollinations between isogenic lines to obtain a suite of genotypes reflecting all possible combinations, would be informative. Extra detail would be needed to ensure the maintenance of the introgression and careful phenotyping would be necessary to capture data that may reflect QTLs with small effects.

3.5 CONCLUSION

The overall objective of this study was to validate putative QTLs in a backcross-derived mapping population, with the long term goal to fine-map and positional clone candidate genes that confer the ability form embryogenic cultures capable of plant regeneration *in vitro*. One advantage of using this population is that the parental genotypes, B73 and A188, are the same as those used to develop the highly responsive parental inbred lines, Hi A and Hi B used to generate the widely used Hi II hybrid (Hi A X Hi B). Another advantage is the contribution of added backcrossing to the maize reference genome genotype, B73, which is the maize inbred line that is most studied in maize genetics research [17]. Therefore, much of the public data on maize refers to the B73 haplotype. But more importantly, the biggest advantage of utilizing the ARC60-derived mapping population is that, through further line development, genotyping, marker-assisted breeding and phenotypic screening, lines developed from this population are much more

similar to the reference genome of B73, or highly isogenic to B73, yet have the ability to produce highly embryogenic, regenerable cultures. Such lines would be very useful for transgenic event functional testing in transformation-based research, as there would be very little confounding effects due to the near isogenic genome background, especially when compared to the HiII hybrid, and transgenes would be tested in a genetic background that has been widely utilized in genetics research and maize breeding.

Table 3.1 A summary of QTL mapping studies in maize tissue culture include the genotypes tested, the population structure, and the phenotype associated with different marker types, the statistical method for QTL detection and the type of molecular markers used in the experiment

Source	Genotype	Population	Phenotype	QTL	Marker type	QTL detection method
[7]	A188 x B73	BC_6S_4	Somatic embryos	1S, 1L, 2, 3, 9L	RFLP	Multiple regression
[7]	A188 x Mo17	F_2	Somatic embryos and plantlets	1, 2, 4, 9L	RFLP	Multiple regression
[15]	FBLL x HiII	BC_6S_4	Plantlets and transformation	1S, 1L, 2L, 3L, 6S, 10S	RFLP, SSR, SNP	Segregation distortion
[20]	H99 x Mo17	F_6 RIL	Type I callus formation	1L, 2L, 3L, 5S, 6S, 8L	RFLP, SSR	PlabQTL with cofactor selection
[19]	18-599 x R15	$F_{2:3:4}$	Embryo culturing capacity	1, 3, 7, 8	SSRs	Mapmaker V.3.0b, QTL Cartographer
[18]	Huangzao4 x Mo17	F_8 RIL	Callus induction, plant regeneration	2, 3, 5, 6, 8, 9	SSR	Composite interval mapping

Table 3.2 Polymorphic single nucleotide polymorphism (SNP) based on the BC_3S_3 ARC60 maize genotype selected and used for initial marker-assisted breeding and near isogenic line development for putative quantitative trait loci associated with tissue culture

SNP	Chromosome	B73 RefGen_v2 position (bp)
PZA03531.1	1	184,088,942
PZB00895.1	1	268,370,364
PZA03692.1	2	166,659,759
PZA03602.1	2	209,504,204
PZA02680.1	2	213,668,709
PZA02456.1	2	210,924,384
PZA02453.1	2	219,554,914
PZA03577.1	2	233,876,337
PZB01944.1	3	7,928,783
PHM2885.31	3	165,901,548
PZA02212.1	3	174,550,727
PHM17210.5	3	178,229,653
PZA03648.1	3	185,317,458
PZA01228.2	3	189,861,328
PZA03743.1	3	191,863,818
PZA03744.1	3	191,864,249
PHM13673.53	3	192,236,275
SYN5782	3	192,262,824
PZE-103137782	3	192,394,349
PZE-103137807	3	192,454,501
PZE-103137948	3	192,648,827
PZE-103138396	3	193,030,215
PZE-103138455	3	193,074,144
PZE-103139795	3	194,146,588
SYN37388	3	194,163,006
PZA01035.1	3	195,407,066
PZA00538.18	3	206,889,455
PZA02457.1	4	29,031,200
PZA03203.2	4	90,203,822
PHM3637.14	4	179,758,341
PZA00694.6	4	235,779,015
PZA03598.1	4	243,936,876
PZA01905.12	4	244,087,450
PZA02239.12	4	244,721,608
PZA01210.1	7	75,099,046
PZA03595.2	9	90,435,061
PZA00062.4	10	146,999,711

Table 3.3 Significant single-marker associations of A188 loci at putative QTL regions in a BC_2S_2 population between ARC60 X B73. Marker positions are derived from the publically available B73 sequence AGP_V2 coordinates.

Marker	Type	Chr	Position[a]	P-value	R^2
PZA03602.1	SNP	2	212,537,417	0.053*	0.11
PZA02456.1	SNP	2	213,948,425	0.009**	0.12
bnlg1160	SSR	3	187,434,674	0.03*	0.08
PZA03744.1	SNP	3	193,609,429	0.01*	0.14
PHM13673.53	SNP	3	194,012,127	0.01*	0.14
PZE-103137948	SNP	3	194,424,679	0.01*	0.14
PZE-103138455	SNP	3	194,803,676	0.02*	0.14
SYN37388	SNP	3	195,941,604	<0.01**	0.21
umc2050	SSR	3	196,073,256	<0.001***	0.21
PZA01035.1	SNP	3	197,247,713	0.04*	0.07
bnlg1605	SSR	3	198,593,706	0.03*	0.08
phi021	SSR	4	13,398,611	0.02*	0.13
PZA02457.1	SNP	4	29,170,943	0.014*	0.1

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 3.4. Maize genotypes representing near isogenic lines (NILs) tested in an experiment to test the effect of putative quantitative trait loci (QTLs) on chromosomes 2, 3 4

Genotype	Line Code	Source	Chromosome	Start position (bp)	End position (bp)
(ARC-60 x B73)28-9-1-52	MAB52	WISN11/32907-52	2	209,493,662	234,681,767
(ARC-60 x B73)28-9-1-54	MAB54	WISN11/32907-54	2	209,493,662	231,524,769
(ARC-60 x B73)30-12-12-94	MAB94	WISN11/32913-94	3	164,683,503	194,277,540
(ARC-60 x B73)30-12-12-94	MAB94	WISN11/32913-94	4	10,877,840	13,789,360
(ARC-60 x B73)30-12-12-84	MAB84	WISN11/32912-84	3	164,683,503	208,947,441
(ARC-60 x B73)30-12-12-84	MAB84	WISN11/32912-84	4	10,877,840	14,510,772

Table 3.5. Randomly selected BC_2F_1 ARC60 families sourced from a 2008 summer nursery that were bulked and sent for doubled haploid seed production

Genotype
(ARC-60 X B73)24-13
(ARC-60 X B73)27-9
(ARC-60 X B73)28-9
(ARC-60 X B73)29-10
(ARC-60 X B73)30-12
(ARC-60 X B73)31-5
(ARC-60 X B73)32-6
(ARC-60 X B73)35-16
(ARC-60 X B73)38-1
(ARC-60 X B73)41-14
(ARC-60 X B73)42-7
(ARC-60 X B73)45-18
(ARC-60 X B73)49-4
(ARC-60 X B73)50-12
(ARC-60 X B73)52-3
(ARC-60 X B73)53-6
(ARC-60 X B73)54-7
(ARC-60 X B73)55-3
(ARC-60 X B73)56-10
(ARC-60 X B73)57-6
(ARC-60 X B73)58-10
(ARC-60 X B73)49-4
(ARC-60 X B73)50-12
(ARC-60 X B73)52-3
(ARC-60 X B73)53-6
(ARC-60 X B73)54-7
(ARC-60 X B73)55-3
(ARC-60 X B73)56-10
(ARC-60 X B73)57-6
(ARC-60 X B73)58-10

Table 3.6. Polymorphic SNPs identified by pooled DNA samples of BC_2S_1 ARC60 families used for marker-assisted breeding of doubled haploid lines for the development of near-isogenic lines with putative quantitative trait loci (QTLs) of interest associated with tissue culture response

SNP	Chromosome	Location	A188	B73
SYN4713	2	210,402,216	G	A
PZE-102172290	2	212,334,746	G	T
SYN38983	2	214,648,399	C	T
SYN21842	2	219,115,236	T	C
PZE-102181538	2	220,938,845	C	T
SYN24940	2	231,388,419	C	T
SYN6923	2	234,681,767	T	C
PZE-103014244	3	7,339,652	C	T
SYN13701	3	8,772,209	C	T
PUT-163a-148995396-718	3	165,901,970	A	G
PZE-103107811	3	167,230,021	T	C
PZE-103126112	3	182,118,368	T	C
PZA03191.1	3	185,290,073	C	T
PZE-103137948	3	192,648,827	C	T
SYN22216	3	194,264,759	G	A
PZE-103140726	3	194,400,487	T	G
PZE-103141647	3	195,402,500	C	T
SYN8972	3	202,574,854	G	A
PZE-103159840	3	209,563,856	T	C
SYN1846	4	179,590,241	A	G
PZE-104141018	4	234,947,078	A	G
PUT-163a-60354034-2731	4	237,089,873	C	G
PZE-104143690	4	238,199,687	T	C
SYN7826	4	243,920,610	C	T
PZE-107000059	7	32,899	C	T
PZE-107001146	7	1,226,282	C	T
PZE-110092938	10	140,869,556	C	T
SYN38569	10	143,258,556	C	A
PZE-110100385	10	144,424,772	A	G
SYN11748	10	146,025,814	T	G

Table 3.7. Maize near isogenic line (NIL) genotypes from marker-assisted breeding and doubled haploid induction that were selected for an experiment to test the effect of putative quantitative trait loci (QTL) on chromosome 3 described by the length of the donor parent, A188, introgression and the estimated centimorgan (cM) distance of the segment the source greenhouse or field nursery and the generation tested

Coded line	Genotype	Source	Generation	Putative QTL (bp)	cM
DH78	W10009_0078	WISN12/008010	DH_1	203,616,720 - 209,563,856	5
MAB485	(ARC-60 X B73)28-9-5-910	WISN11/002010-910	$BC_2F_{2:3}$	187,789,641 - 204,871,368	12
DH99	W10009_0099	WISN12/008011	DH_1	164,683,503 - 188,705,744	13
DH240	W10009_0240	WISN12/008020	DH_1	164,683,503 - 199,472,604	20
MAB94	[B73 X [(ARC-60 x B73)30-12]]-12-94	WISN11/32913-94	BC_3F_2	164,683,503 - 194,277,540	20
MAB417	[B73 X [(ARC-60 x B73)30-12]]-12-78	WISN11/32912-78	BC_3F_2	177,662,596 - 207,539,405	22
MAB84	[B73 X [(ARC-60 x B73)30-12]]-12-84	WISN11/32912-84	BC_3F_2	164,683,503 - 208,947,441	30

Table 3.8 Summary statistics and test for normality for plantlet regeneration response in a greenhouse experiment testing the effect of putative quantitative trait loci associated with tissue culture response in maize near-isogenic lines harboring either one or two putative QTLs

N	35	Sum Weights	35	
Mean	4.43	Sum Observations	155	
Std Deviation	4.05	Variance	16.43	
Skewness	1.52	Kurtosis	3.46	
Uncorrected SS	1245	Corrected SS	558.571429	
Coeff Variation	91.52	Std Error Mean	0.69	

Tests for Normality

Test	Statistic		p Value	
Shapiro-Wilk	W	0.86	Pr < W	0.0004***
Kolmogorov-Smirnov	D	0.21	Pr > D	<0.0100**
Cramer-von Mises	W-Sq	0.22	Pr > W-Sq	<0.0050**
Anderson-Darling	A-Sq	1.31	Pr > A-Sq	<0.0050**

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 3.9 A one way non-parametric Kruskal-Wallis test to determine differences in genotypes using a Chi-square test and also an estimate of Wilcoxon Score rank sums for each genotype

Kruskal-Wallis Test	
Chi-Square	20.4377
DF	8
Pr > X^2	0.0088**

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Wilcoxon Scores (Rank Sums)

NIL	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
MAB52	3	18	54	16.77	5.83
MAB54	1	4	18	9.98	3.50
MAB84	2	7	36	13.91	3.50
MAB94	4	93	72	19.06	23.13
A188	7	184	126	23.97	26.29
ARC60-26	5	124	90	20.97	24.80
ARC60-28	5	97	90	20.97	19.30
B73	4	49	72	19.06	12.25
HiII	4	56	72	19.06	14.00

Table 3.10 A t-test between two groups of maize near isogenic lines designated as monogenic or digenic harboring either one or two putative quantitative trait loci (QTL) associated with tissue culture response measured as the average number of plantlets regenerated per zygotic embryo tested

Summary statistics

Type	N	Mean	Std Dev	Std Err	Minimum	Maximum
Digenic	6	5.5	7.45	3.04	0	19
Monogenic	4	0.5	1	0.5	0	2

T-test

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	8	1.31	0.2272
Satterthwaite	Unequal	5.27	1.62	0.1627

F-test Equality of Variances

Method	Num DF	Den DF	F Value	Pr > F
Folded F	5	3	55.50	0.0074

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 3.11 Summary statistics and test for normality for plantlet regeneration response in a greenhouse experiment testing the effect of putative quantitative trait loci associated with tissue culture response in maize near-isogenic lines harboring putative QTLs on chromosome 3

N	52	Sum Weights	52
Mean	25.38	Sum Observations	1320
Std Deviation	41.86	Variance	1753
Skewness	2.09	Kurtosis	3.23
Uncorrected SS	122886	Corrected SS	89378
Coeff Variation	164.92	Std Error Mean	5.81

Tests for Normality

Test	Statistic		p Value	
Shapiro-Wilk	W	0.63	Pr < W	<0.0001***
Kolmogorov-Smirnov	D	0.28	Pr > D	<0.0100**
Cramer-von Mises	W-Sq	1.44	Pr > W-Sq	<0.0050**
Anderson-Darling	A-Sq	7.72	Pr > A-Sq	<0.0050**

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 3.12 Summary statistics and an analysis of variance (ANOVA) on plantlet regeneration response in tissue culture in a greenhouse experiment to test the effect of a putative quantitative trait loci on chromosome 3 associated with tissue culture response in near isogenic lines in maize genotypes planted in replicates at different planting times.

N	52	Sum Weights	52
Mean	25.38	Sum Observations	1320.00
Std Deviation	41.86	Variance	1752.52
Skewness	2.09	Kurtosis	3.23
Uncorrected SS	122886.00	Corrected SS	89378.31
Coeff Variation	164.92	Std Error Mean	5.81

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	1	1885.42	1885.42	12.88	0.0021**
gh	1	12.54	12.54	0.09	0.7730
rep(gh)	4	627.54	156.89	1.07	0.3992
genotype	9	43413.29	4823.70	32.96	<.0001***
time*gh	1	81.13	81.13	0.55	0.4661
time*genotype	7	17730.40	2532.91	17.31	<.0001***
gh*genotype	6	1188.84	198.14	1.35	0.2853
time*gh*genotype	3	272.75	90.92	0.62	0.6103

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 3.13 An analysis of variance and the least squares means of genotypes tested in a single greenhouse experiment to test the plantlet regeneration response in near isogenic lines in maize

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	49417.51	2906.91	10.14	0.0012**
Error	8	2294.03	286.75		
Corrected Total	25	51711.54			

R-Square	Coeff Var	Root MSE	Mean
0.96	64.37	16.93	26.31

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	1	500.16	500.16	1.74	0.2231
rep(time)	4	587.80	146.95	0.51	0.7291
genotype	6	27288.83	4548.14	15.86	0.0005***
time*genotype	6	10317.16	1719.53	6.00	0.012*

Genotype	LSMEAN	Standard Error	Pr > |t|
ARC-60	92.17	8.11	<.0001***
B73	1.94	11.40	0.8690
DH240	24.19	11.40	0.0665
DH78	-5.47	11.36	0.6428
DH99	8.83	6.91	0.2372
MAB417	8.62	10.64	0.4414
MAB485	18.93	13.37	0.1945

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 3.14 A one way non-parametric Kruskal-Wallis test to determine differences in genotypes using a Chi-square test and also an estimate of Wilcoxon score rank sums for each genotype

Kruskal-Wallis Test	
Chi-Square	33.05
DF	9
$Pr > X^2$	0.0001***

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Wilcoxon Scores (Rank Sums)

Genotype	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DH78	7	59.5	185.5	36.75	8.50
B73	5	51.0	132.5	31.74	10.20
MAB94	5	104.5	132.5	31.74	20.90
DH99	9	235.5	238.5	40.73	26.17
DH240	5	136.0	132.5	31.74	27.20
MAB417	6	169.5	159.0	34.40	28.25
MAB485	2	71.5	53.0	20.70	35.75
MAB84	3	108.5	79.5	25.10	36.17
ARC-60	9	395.0	238.5	40.73	43.89
A188	1	47.0	26.5	14.79	47.00

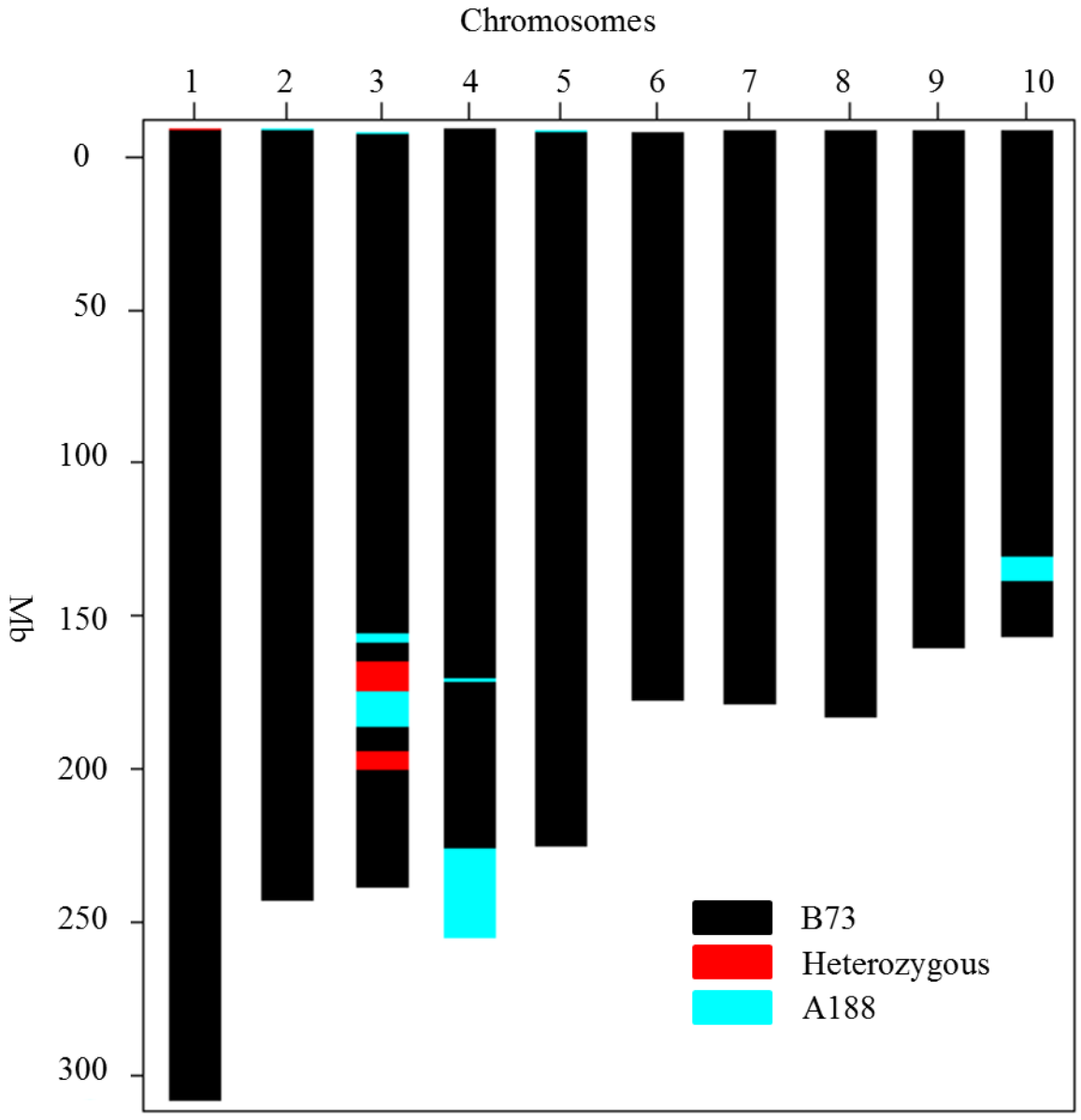

Figure 3.1. A karyotype of the BC_3S_3 ARC60 maize inbred line using 55,000 single nucleotide polymorphisms (SNP) genotyping across all ten chromosomes

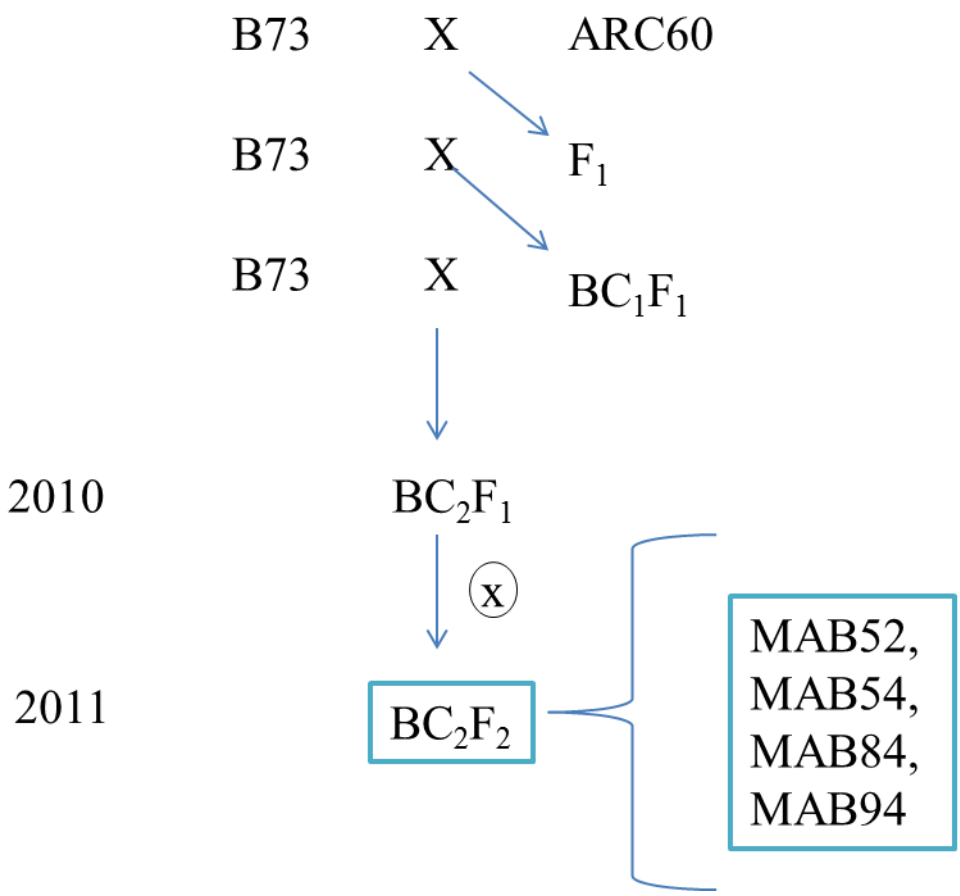

Figure 3.2. Population development to identify near-isogenic lines using marker-assisted breeding to generate monogenic NILs MAB52 and MAB54 that harbor a single putative QTL associated with tissue culture response from the maize inbred line A188 with a single introgression on chromosome 2 population development for digenic NILs MAB84 and MAB94 that harbor 2 putative QTLs with A188 introgressions on chromosomes 3 and 4

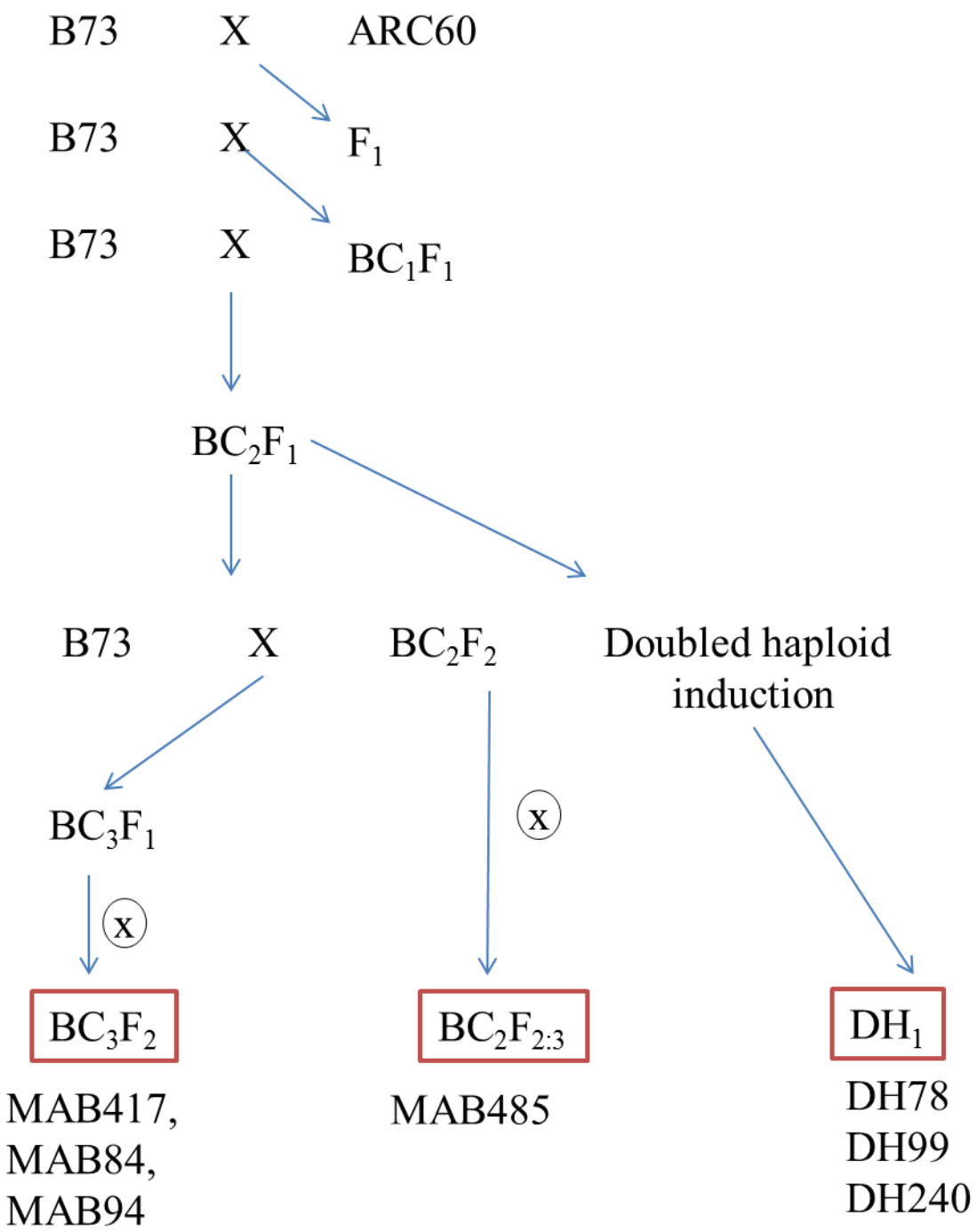

Figure 3.3. Population development to identify near-isogenic lines using marker-assisted breeding to generate lines with putative QTLs on chromosome 3 associated with tissue culture response

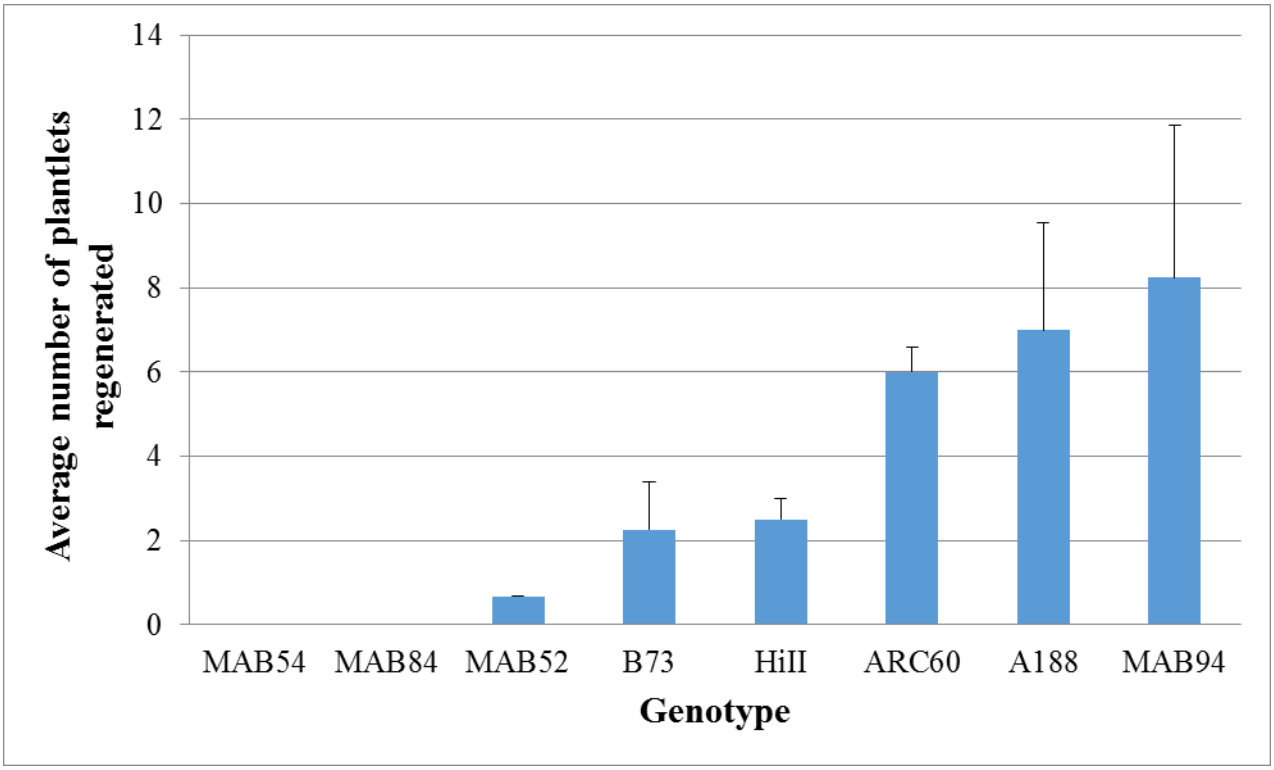

Figure 3.4. Raw data on plant regeneration response (PR) in tissue culture comparing NILs with either one or two A188 introgressions to test the effect of putative QTLs on chromosomes 2 and 3 in monogenic and digenic near-isogenic lines (NILs)

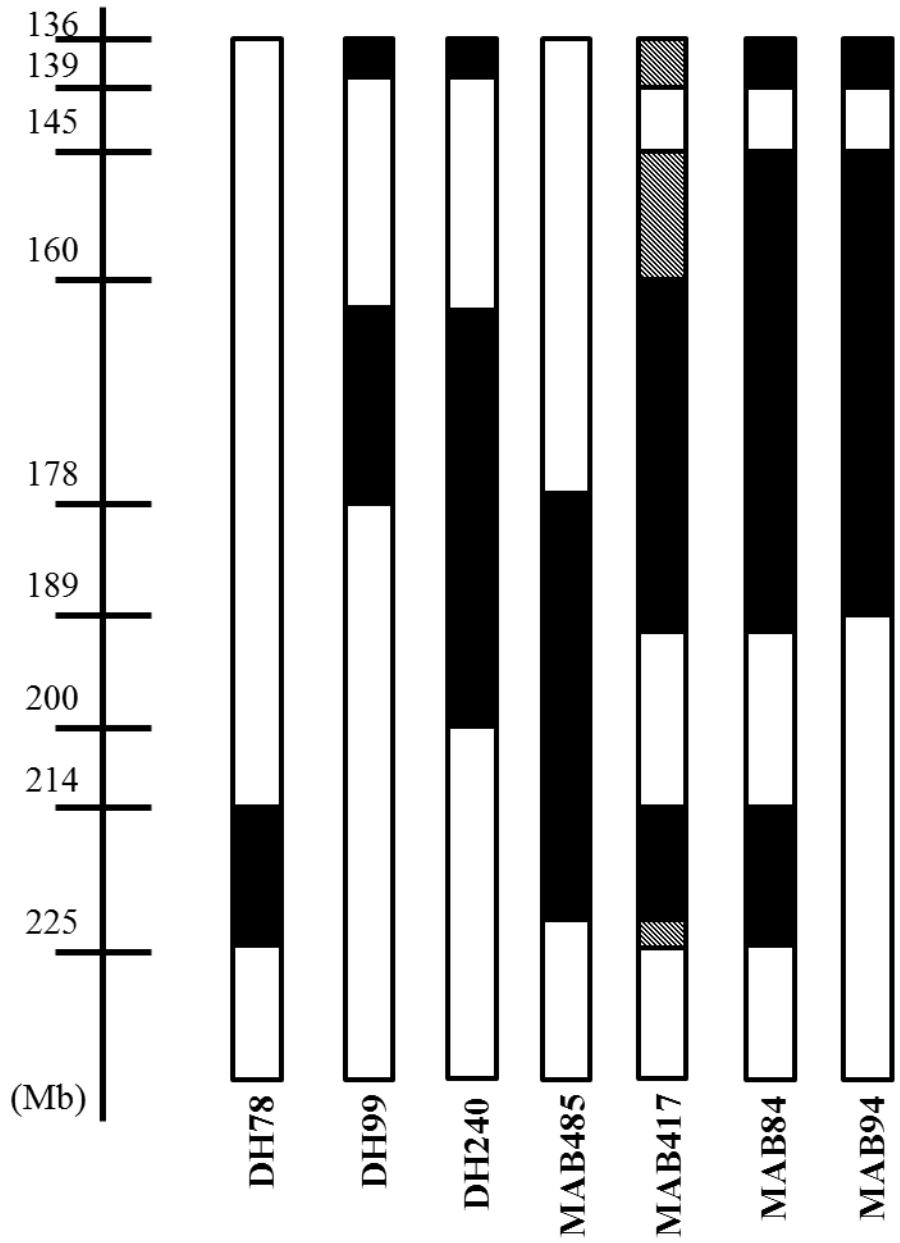

Figure 3.5. A diagram of the physical length of A188 introgressions in megabases (Mb) as determined by markers mapped to the B73 reference genome on chromosome 3 in near isogenic line (NIL) genotypes DH78, DH99, and DH240 which are doubled haploids, MAB485 and MAB417 which were developed with marker-assisted breeding (MAB). A188 segments are black, B73 segments are white, and heterozygous regions are in grey. MAB94 and MAB 84 which were also developed by MAB but have an additional A188 introgressions on chromosome 4. These NILs were selected for initial study to test the effect of putative QTLs on chromosome 3.

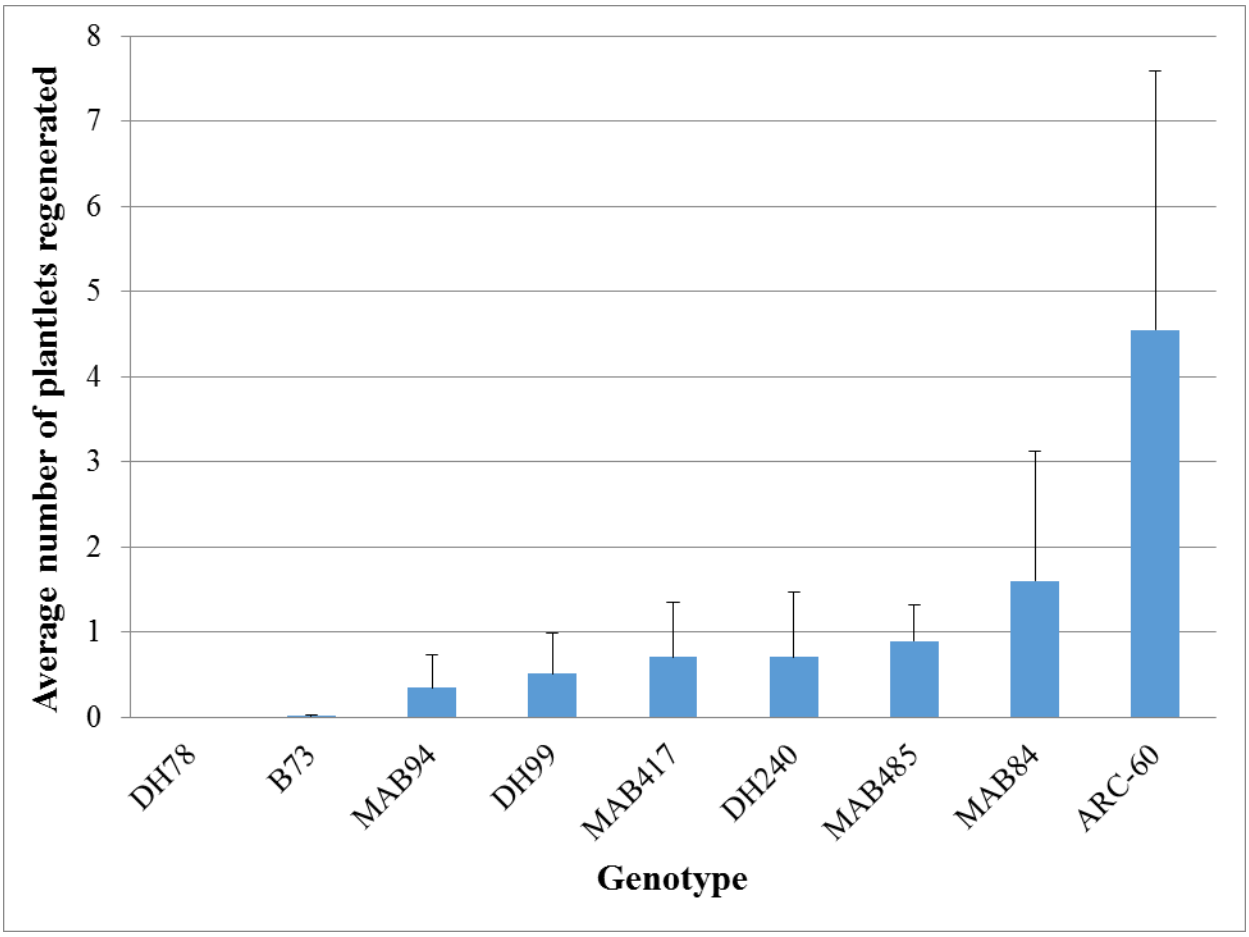

Figure 3.6. Average number of plantlets regenerated for each near isogenic line genotype tested in a greenhouse experiment to validate the effect of a major putative QTL on chromosome 3

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CHAPTER 4: FINE MAPPING FOR CANDIDATE GENES ASSOCIATED WITH TISSUE CULTURE RESPONSE IN MAIZE

4.1. INTRODUCTION

Embryogenic tissue cultures have been utilized for research on a wide range topics including biochemistry, cellular, and molecular biology of the initial stages of zygotic embryo and seed development through germination, all of which has allowed for the development of applications of tissue culture-based methods in improving processes and products in crop plants such as clonal plant propagation and genetic engineering. Genetically engineered seeds are an integral part of the global food and feed system in the United States (US) and world-wide [1]. In 2013, 169 million acres of transgenic corn, cotton and soybeans were grown in the US, accounting for nearly half of the total arable, farmed land in the US, whereas acreage planted to transgenic corn was 87.64 million acres, or 90% of the total corn acres in the US [2]. One important process for genetic engineering-based applications is the ability to regenerate plants *in vitro* such that somatic cells that have been transformed can transition into embryogenic callus that produces somatic embryos capable of developing into fertile plants. The ability to form embryogenic regenerable tissue cultures is genotype dependent for maize and other crop species. This limitation hinders tissue culture-based research in clonal propagation and genetic engineering applications. When the desirable outcome is to generate seed for downstream processes in crop development for genetically modified (GM) organisms and functional genomics testing in plant science research, a deeper understanding of the genes involved in this process would enable expanding this technology to other genotypes in maize or other plant species that are also limited to genotype specificity in tissue culture.

For maize, a globally important crop and model species for crop genetics and tissue culture research, functional genomics research and the introgression of transgenic traits into elite inbred lines for commercial hybrid production involves transgene delivery into a donor line genotype having high embryogenic, regenerable tissue culture response. This donor line is transformed *in vitro* to contain the genes of interest, and therefore, it must be amenable to the tissue culture process and capable of regeneration ability. The donor plant must be able to develop into a whole plant where the transformed seed can then be used in subsequent crossing to desired genetic stocks or conversion of elite inbred lines. In maize tissue culture, a common target genotype for genetic engineering is the maize "Hi II" germplasm, a hybrid cross between lines designated as "Parent A" and "Parent B" [3]. Hi II was generated from a cross between the highly embryogenic, regenerable maize inbred line, A188, to the maize inbred line, B73, the source of the sequenced maize reference genome and a line widely utilized in early maize breeding programs in the US. Both A188 and B73 belong to the Iowa Stiff Stalk heterotic group [4], but are very different physiologically and agronomically. A188 flowers early and its seeds are white and pointed. It is, however, highly amenable to the tissue culture process generating with a 100% efficiency in embryogenic callus cultures per zygotic immature embryo plated in tissue culture [4]. Maize inbred line B73 is the genotype sequenced to generate the initial reference genome for maize [5]. It flowers early –to-mid in the growing season and has yellow dent seeds. B73 is taller and has better agronomic characteristics than A188 [6]. Unfortunately, maize inbred line B73 has a very low embryogenic, regenerable tissue culture response with, for example only a 2% efficiency [3] in immature zygotic embryos capable for generating green plantlets. Two lines, Parent A and Parent B, were selected for high tissue culture response and improved plant vigor from an F_2 population derived from the cross of A188 and B73 [3]. When

crossed and selfed, the resulting F_1 seed is planted, pollinated and the F_2 embryos are then utilized for efficient embryogenic culture initiation with the added benefit of possessing a genotypic background that is more similar to the reference genome of maize inbred line B73. Hi II is highly efficient in forming embryogenic callus and green plantlets *in vitro*, but it has some disadvantages for use in genetics research and maize crop improvement applications. Because Hi II is a hybrid, when conducting functional genomics testing via transformation, it is difficult to determine if the phenotypes displayed are due to the transgene or is confounded by the high levels of heterozygosity between F_2 source embryos. The most advantageous source for maize transformation and for functional genomics research is to have a near isogenic line (NIL) that is highly similar in genotype to the maize reference inbred line B73, but is also highly capable of regeneration ability and amenable to the tissue culture process.

It has been suggested that embryogenic, regenerable tissue culture response is controlled by only one to a few major genes with large effects [6]. Previous studies have eluded tissue culture response as additive genetic variation displayed in the effect of parental genotype in the initiation of embryogenic callus [7] and regeneration ability [4]. Tissue culture response was shown to improve through backcross breeding and marker-assisted selection in maize, suggesting that a major gene or genes is or are responsible for tissue culture traits [6].

The specific genes involved in the onset of somatic embryogenesis and regeneration ability in plant species are not yet fully understood, and there has been only one study to date that has successfully identified a candidate gene by quantitative trait loci (QTL) mapping [8]. Even then, this single gene which was associated with regeneration ability in rice is not widely used in improving transformation or regeneration ability in rice or in other plant species [9]. In the rice study, the QTL approach identified a difference in nitrite metabolism between the

parental lines in the mapping populations that was also associated with differences in tissue culture response, but when looking at the diversity of rice germplasm, most rice plants already have a high nitrite metabolism capacity such that the gene associated with tissue culture response has little functionality in those genetic backgrounds, suggesting that there are probably other genes involved in the control of tissue culture response [10].

There are only a handful of studies in plants that have successfully cloned candidate genes within QTLs, and the most common approach in cloning has been through positional cloning, or map-based cloning using near isogenic lines (NILs) to verify QTLs and fine-map for candidate genes [11]. NILs offer the added benefit of testing the effect of the QTL in a highly homozygous background with reduced genetic variability that minimizes the effect of genetic variation due to background [12]. Although NILs have been suggested to improve QTL discovery, based on simulations, using a NIL population structure is not the most ideal population structure for initial QTL detection [12]. Nonetheless, attempts to simultaneously verify and fine-map QTLs using introgression libraries [13] and positional cloning [11] have shown merit in identifying causal genes.

QTL studies have been conducted in maize for tissue culture response [6,14-17], but few have attempted to fine-map QTLs for tissue culture traits in maize. The objective of this research was to conduct fine-mapping of a backcross-derived, doubled haploid, maize inbred line that is near-isogenic to the maize reference inbred, B73, but harbors a small segment on chromosome 3 from the maize inbred donor line, A188, that has a high embryogenic, regenerable tissue culture response. The long-term goal of the research is to identify candidate genes for embryogenic, regenerable tissue culture response by map-based cloning. The goals of this study were to (1) improve the genetic and physical resolution of QTL mapping for chromosomal regions that are

associated with embryogenic, regenerable tissue culture response in maize and (2) identify closely linked SNP markers for marker-assisted selection of improved donor lines for use in tissue culture research.

4.2. MATERIALS AND METHODS

Plant materials

The maize inbred line, B73, is an Iowa Stiff Stalk line with good agronomic performance and yellow dent seeds. The experimental line, DH99, was developed at the University of Wisconsin from doubled haploid induction of BC_2S_1 seeds of an ARC60 X B73 backcross-derived mapping population (Figure 4.1). ARC60, a BC_3S_3 line, was generated from a cross between maize inbred line A188 and B73 (Appendix D). The DH99 line is near-isogenic to B73, but contains two small segments of the maize inbred line A188 located on chromosome 3. In a previous study, DH99 displayed relatively moderate to high embryogenic, regenerable tissue culture response, making this line an ideal source for fine-mapping for candidate genes related to efficient tissue culture response. A cross between maize inbred lines B73 and a near-isogenic, doubled haploid line, DH99, was made in the greenhouse in fall of 2012 at the University of Wisconsin Walnut Street Greenhouse in Madison, Wisconsin. The F_1 seed was planted in the greenhouse on January 18, 2013. Plants were self-pollinated in 2013 to generate segregating F_2 seed. Flanking markers to identify informative crossovers in the QTL region were utilized to screen 2,243 F_2 seeds. Select plants were self-pollinated. In February 2014, F_3 seeds were planted in the greenhouse and represented homozygous plants now segmented for the QTL region of interest.

Greenhouse growing environment

Plants for this experiment were grown in the greenhouse at the University of Wisconsin Walnut Street Greenhouse Complex (Madison, WI). Single plants were grown to maturity in classic 2000 pots (19:3 L; Hummert International, Earth City, MO). Pots were prepared with Scotts MetroMix 360 soil (The Scotts Company, Marysville, OH) and 32 g of Osmocote Plus 19-5-8 controlled-release fertilizer (Hummert International, Earth City, MO). Seeds were placed in pots full of metromix and drenched with water. After sowing single seeds one inch from the soil surface, pots were watered lightly for to cover the seed with soil and to ensure ample soil compaction and then left without watering again for approximately one week. After emergence, plants were watered every two days to every other day until after the fourth-leaf stage of plant development. At the V8 stage of physiological maize development, when the plants had nearly 8 leaves, the pots were treated with liquid fertilizer twice a week. Greenhouse fertilizer mixed with water delivered 350 ppm of nitrogen and contained 20-10-20 Peters Professional (The Scotts Company, Marysville, OH) water soluble fertilizer with elevated levels and chelated micronutrients. Plants were grown under natural light and supplemental lighting conditions year round (16 hr light: 8 hr dark). The average light level one meter from the greenhouse floor was 580 µmoles from artificial light provided by 1000 watt high pressure sodium bulbs. Temperatures were maintained at 28^{o}C. Ear shoots were covered after emergence and controlled pollinations were made to ensure self-pollinating of each plant entry. Four weeks after pollinations, husks were pulled back and allowed to dry and watering ceased. Plants were left to dry out for an additional 3 weeks until harvest. Three different greenhouses were used for this study. Two greenhouses had two different planting times two weeks apart. The first plantings were arranged in a completely randomized design with 2 replicates per source ear planted in each greenhouse. Due to poor germination, a second planting time was necessary to replant source

seeds that did not germinate. In the third greenhouse, only one planting was done, also planted as a completely randomized design with 2 replicates per source ear.

Tissue culture

In order to assess tissue culture response in this study, immature zygotic embryos were harvested from greenhouse grown plants and used as explants to initiate tissue culture growth. Approximately 12 days post pollination when immature embryos were 1.2-1.5 mm along the longitudinal axis from end to end, half of the ear was harvested. The remaining cob was left to develop into mature seeds for the next generation. The harvested portion of the ear was prepared for tissue culture by sterilizing for 20 minutes in a 50% bleach and sterilized water solution containing a drop of Tween-20 followed by three washes with sterile water. All work was conducted in a sterile, laminar flow hood. A total of 50 immature embryos per ear was isolated and placed scutellum side up (embryo axis side down) onto two plates (25 embryos per plate) containing initiation/maintenance (IM) medium with the following formulation autoclaved for sterilization for 30 minutes and allowed to cool before pouring onto plastic 100 mm x 25 mm 2 inch petri plates: N6 salts [18] and 1000 X vitamins [1g/L thiamine-HCL, 0.5g/L pyridoxine-HCL, 0.5g/L nicotinic acid, and 2g/L glycine], 2.0 mg/L 2,4-D, 2.875g L-proline, 30g sucrose, 8.5 mg/mL or 50 µM $AgNO_3$ (Appendix B). Plates were wrapped with micropore tape and placed in a dark growth chamber for 10 days in the dark at 28^{o}C. At 10 days, the growing embryonic axis was excised and the embryos were transferred onto fresh initiation medium. Callus was transferred onto fresh media every two weeks for a total of 38 days on initiation medium where, after the second subculture, only the 10 highest responding embryos based on callus diameter and embryogenic mass were selected for continued subculture. The embryogenic tissue was then transferred to Regeneration Medium 1 (RM1) consisting of 4.3 g/L Murashagie

and Skoog salts [19], 60 g/L sucrose, 0.1 g/L myo-inositol, 1 mL/L 1000X MS vitamin stock [0.5 g/L thiamine-HCL, 0.5 g/L pyridoxine-HCL, 0.05 g/L nicotinic acid, and 2 g/L glycine] (Appendix B). RM1 was prepared by autoclaving for 30 minutes and allowed to cool to 65-70 degrees C. Filter sterilized stock solutions were then added to the RM1 including 1 mg/L IAA, 0.5 mg/L zeatin, and 0.023 mg/L ABA, and the medium was poured into sterile, 100mm x 25mm plastic petri plates. RM1 plates containing the transferred tissues were kept in an incubator in the dark at 28^{o}C for 10-14 days. The tissue was then transferred onto Regeneration Medium 2 (RM2) consisting of 4.3 g/L MS salts, 40 g/L sucrose, 0.1 g/L myo-inositol, 1 mL/L 1000X MS vitamin stock [0.5 g/L thiamine-HCL, 0.5 g/L pyridoxine-HCL, 0.05 g/L nicotinic acid, and 2 g/L glycine], and hormone stock solutions at 1mg/mL (1 mL/L IAA, 0.5 mL/L zeatin, 23.2 µL/mL ABA) (Appendix B). Plates containing transferred tissues were stored in an incubator at 26^{o}C with 16 hr light: 8 hr dark photoperiod to allow for shoot formation. Light intensity (at 100-150 µmol $m^{-2}s^{-1}$) was supplied by 17 watt Phillips F17T8 bulbs set at 16 cm above the plates. All media was pH 5.8 and solidified with 3.5g/L Gelzan. Tissue was kept on RM2 for 14 days.

Genotyping

In order to determine single seed sources as homozygous recombinant types, six SNP markers (Table 4.1) were used to genotype 2,243 F_2 seeds using the KASPar genotyping assay [20]. Seed chips were taken using guillotine style dog nail clippers and then, with forceps, chips were placed into 96-well plates for DNA extraction using the sbeadx® maxi plant kit (cat. no. 41602, LGC Genomics, Berlin, Germany). The DNA extraction method was modified for maize seed chips (Appendix A.3). The seed chips were sent for extraction and genotyping by LGC Genomics genotyping services (Boston, MA).

Phenotyping

Tissue culture traits were measured at two time points representing early stage somatic embryogenesis and late stage somatic embryogenesis (Figure 4.2). Measurement of early somatic embryogenesis traits was done at 24 days after plating on tissue culture initiation medium to aid in the visual selection of callus lines maintained in tissue culture for the remainder of the experiment. After measuring callus diameter and counting the number of zygotic embryos displaying somatic embryogenesis, ten zygotic embryos displaying the highest growth rate and embryogenic response were visually selected and subcultured onto maintenance medium , and callus lines derived from ten zygotic embryos displaying the highest growth rate and embryogenic response were visually selected and. The number of zygotic embryos displaying somatic embryogenesis was counted by visual appearance of embryogenic structures protruding from the callus mass (Figure 4.3). Typically, there were 50 zygotic embryo explants isolated per source ear, and embryos were placed on tissue culture initiation medium at a rate of 25 embryos per plate. For each plate, callus derived from each of three randomly selected zygotic embryos was measured for callus diameter and the average diameter of the three samples was calculated. For late somatic embryogenesis trait analysis, the number zygotic embryos producing callus that displayed somatic embryogenesis was counted again. This count data was taken 38 days after plating on tissue culture to determine if the early visual ratings of somatic embryogenesis were maintained. Late callus diameter was measured by visually selecting the three largest callus masses per plate representing independent callus lines derived from independent zygotic embryos. The average was calculated for the three samples for each plate. The total number of green plantlets regenerated (PRL) was also counted at the end of the experiment at 14 days after transfer onto regeneration 2 medium.

In addition to phenotypic traits regarding tissue culture response, opportunistic agronomic traits were also taken in the greenhouse. The number of ear shoots was counted for each plant. The ear height was measured in centimeters from the bottom of the pot to the bottom of the primary ear node. The plant height was measured in centimeters from the bottom of the pot to the bottom of the flag leaf node. Finally, the number of tillers was counted to not include the primary stalk, but only counting auxiliary stalks.

Data analysis

Data analysis was conducted using SAS v9.3 (SAS Institute, Cary, NC) (Appendix C.2). Pearson's correlations for tissue culture and agronomic traits were calculated. The statistical test for normality was conducted in addition to visualizing histograms, probability plots and normal quantiles plots to visually inspect data distribution. To attempt to overcome the non-normal distribution for tissue culture trait data an ln(x+1) transformation was done prior to data analysis to reduce effects of extreme outliers [21], however, the transformation did not show normalized data, so the remainder of the study reports test statistics on raw data. The Kruskal-Wallis test statistic was used to determine if there were significant differences between genotypic type means. A Wilcoxon scores for rank sums for each tissue culture trait using the genotypic type as a variable was used to rank trait means from high to low phenotype. In addition, an analysis of variance was done to transformed data to determine sources of variation in the experimental design. The following model was used:

$$Y \sim \mu + G + T + L + R(L) + TxL + GxT + GxL + GxTxL + \varepsilon$$

Where Y is the observed trait measured as the square root of the number of plantlets regenerated per plant as a sum from the 20 best selected zygotic embryos for each plant, to determine

regeneration ability or tissue culture response. μ is the overall mean, G are genotypes, T is planting time, L is greenhouse location, R is replicates which are nested within greenhouse location. In addition, the following interactions were accounted for in Model I: TxL is interaction between planting time and greenhouse location, GxT is the interaction between genotype and planting time, GxL is the interaction between genotype and greenhouse, and GxTxL is the interaction between genotype, planting time, and greenhouse location. Only sources of variation showing significance at ($P<0.05$) were deemed as significant. All other non-significant sources of variation were dropped from the analysis. Finally, single marker-trait associations were determined for each SNP marker and raw tissue culture trait by running linear model to obtain the mean and standard deviation for each allelic class , the F-value, the probability statistic as the p-value, and also the R^2 representing the phenotypic variability explained by the marker. Additional annotations were determined by MapMan genome release for *Zea mays* based on B73 5b filtered gene sets (http://mapman.gabipd.org/)

4.3. RESULTS

High density genotypic information reveals genomic differences between Hi II and the ARC60 backcrossed derived mapping lines

High density genome-wide SNP information was used to compare test lines and controls with the 55,000 SNPs on the MaizeSNP50 Illumina SNP chip (Illumina, San Diego, CA). This assay resulted in the identification of A188 introgressions and polymorphic SNP markers to aid in fine-mapping. A comparison between A188 and B73 alleles in the ARC60 parental line, the Hi II hybrid, and a select doubled haploid line, DH99, revealed the percentage of A188 and B73-derived alleles in each source. When considering only informative markers that were

polymorphic between A188 and B73, out of 21,881 SNP markers, the proportion of the genome similar to B73 in the HiII hybrid, a common source for embryogenic callus production in maize, is 69.11% (Figure 4.4). ARC60, which is 97.54% similar to B73, retained 7 regions of A188 introgressions (Appendix C). DH99 had only two small regions on chromosome 3 between 164,821,641 and 166,794,453 base pairs (bp) and another between 178,772,856 and 188,705,744 bp and is 99.22% B73 (Figure 4.5).

Genotypic screening of single seed recombinants to determine recombination frequency between SNPs loci

In order to study the effect of recombinant genotypes for the putative QTL of interest, 2,243 single F_2 seeds from 62 source ears were screened for recombinant homozygous genotypes. Thirty-six single seeds from each source ear were genotyped with six SNP markers (Table 4.1). A total of 481 single seeds were homozygous recombinants. Using the genotyping information to determine the number of recombinants in each interval, the recombination frequency between SNP markers was determined, giving an estimate of genetic distance in centimorgans (cM) (Table 4.2). The DH99 NIL had two segments of A188 on chromosome 3. One segment was between PZE-103105125 and PZE-103107449 that, based on recombination frequency, had a genetic distance of 2 cM, with an estimated 45 genes in that interval. The second A188 segment was between PZE-103122471 and PZE-103135061 with a 10 cM genetic distance between the two flanking SNP, with an estimated 288 genes in that interval.

DH99-derived recombinants screened for fine-mapping experiment

A total of 296 homozygous QTL-NIL recombinant genotypes were identified and planted in three greenhouses at varying planting times, with two replicates per seed coming from the

same source ear. Poor germination due to the seed chipping procedure resulted in growth to maturity of only 129 plants for phenotyping. Plants that were tested represented 15 genotypic types (Figure 4.6) with 1-20 replicates per genotypic type tested (Table 4.3). A total of 47 different source ears were tested for the effect of putative QTLs on chromosome 3 on embryogenic, regenerable tissue culture response, with 21 source ears with only one successful replicate and 26 source ears with greater than 2 and up to 7 source replicates per control. For example, the genotypic type "B" was tested for the effect of tissue culture response with 12 different source ears of which 6 source ears had only one replicate and the other 6 source ears had greater than 2 source replicates up to 5 replicates successfully tested (Table 4.3). Four control lines A188, B73, HiII, and ARC60 were successfully tested with greater than 3 replicates each.

Summary statistics

Tissue culture traits were measured at two different time points designated as early or late (Table 4.4). Early callus diameter and number zygotic embryos visibly showing somatic embryogenesis were measured at 24 total days in IM media. Late callus diameter and number zygotic embryos visibly showing somatic embryogenesis were measured after 38 days in culture on IM medium, right before callus was transferred to RM1. The mean early callus diameter across all genotypes tested (N=128) was 0.47 cm with a standard deviation of 0.23. Minimum and maximum early callus diameters ranged from 0.24 cm to 1.47 cm. The mean late callus diameter was 1.05 cm with a standard deviation of 0.79. Minimum and maximum late callus diameters ranged from 0.32 cm to 3.53 cm. The mean proportion of zygotic embryos producing callus with somatic embryos in early and late measurements was 30.7% and 30.3%, respectively. The number of plantlets regenerated had mean count number of 12.31 with a standard deviation

of 21.91. The maximum number of plantlets regenerated was 103 (Table 4.4). Opportunistic agronomic traits measured on basic plant morphology were also summarized (Table 4.4). Plant ear height had a mean value of 97 cm ranging from 49 to 140 cm. Plant height had a mean value of 200 cm ranging from 112 to 249 cm. Mean number of ears and shoots was 3 and ranged from a minimum of one to a maximum of 6. Number of tillers had a mean of 1with a minimum of 0 to a maximum of 4.

Correlations

Statistical correlations were calculated among tissue culture traits (Table 4.5). Correlations between early and late tissue culture traits were highly significantly correlated ($P < 0.001$) with early callus diameter and late callus diameter correlated at 0.7736 and early and late somatic embryogenesis correlated 0.8980. Number of plantlets regenerate was also significantly correlated ($P<0.0001$) with early somatic embryogenesis, late somatic embryogenesis and late callus diameter at 0.5948, 0.574, and 0.7234, respectively. Early callus diameter was significantly correlated 0.2205 ($P>0.012$).

Test for normality and significant sources of variation in experimental design

The Shapiro-Wilk statistical test was used to test the assumption for normally distributed data. The test statistic was significant ($P<0.0001$) for all tissue culture traits suggesting a non-normal data distribution (Table 4.6). An analysis of variance was conducted on raw data set, knowingly violating the assumption for normally distributed data. The full model was conducted to determine sources of variation and interactions between variables in the experimental design. Based on Type II sums of squares, only genotypic type was shown to be significant ($P<0.0001$)

for early and late tissue culture traits, however, for the number of plantlets regenerated, the planting time by greenhouse by genotype interaction was also significant (P>0.0453) (Table 4.7)

Differences in embryogenic, regenerable tissue culture response among genotypes

Since the dataset is non-parametric, this study required the use of a test that does not make assumptions about normality that can be performed to determine statistical differences between genotypic means. A Kruskal-Wallis that challenges the assumption of equal trait means was conducted for all culture traits, whereas all test for early and late tissue culture traits and plantlets regenerated was highly significant (P<0.0001) indicating that one or more means between genotypic types were indeed different. In addition, a statistical analysis to rank genotypic means from high to low was done using a Wilcoxon rank (Table 4.8) of genotypic types for all tissue culture traits. Visual inspection of raw data values and standard deviations were plotted (Figure 4.7 A-E) showing the differences in tissue culture performance between genotypes.

Test to determine fine-mapping segment associated with embryogenic, regenerable tissue culture response

A linear model was used to determine significant associations between marker genotypes and raw phenotypic tissue culture trait data as early and late callus diameter and early and late somatic embryogenesis. Indeed, in all marker trait associations, the A188 allele was consistently associated with a higher mean value for all tissue culture traits. Two SNP markers PZE_103122471 mapped to a location of 178,772,856 bp on chromosome 3 and SYN29001 mapped to 181,826,658 bp were consistently significantly associated with phenotypic tissue culture trait differences between A188 and B73 genotypes (P< 0.0001) explaining 18% to 41%

of the phenotypic variation (Table 4.9). Four SNPs were highly significantly associated with early callus diameter: again PZE_103122471 and SYN29001 were significant (P<0.0001), and PZE_103133772 and PZE_103135061 were significant at (P >0.0001) and (P>0.0004), respectively. PZE_103122471, SYN29001, and PZE_103133772 were highly significantly associated (P<0.0001) with early somatic embryogenesis. Four SNP markers PZE_103122471, SYN29001, PZE_103133772, and PZE_103135061 were highly significantly associated with late callus diameter and late somatic embryogenesis (P<0.0001). Number of plantlets was highly significantly associated with PZE_103122471 and SYN29001 (P<0.0001) with an R^2 of 20.5% and 17.4%, respectively (Table 4.9).

4.4. DISCUSSION

Early tissue culture phenotypes can be used to determine efficient embryogenic capacity

Early somatic embryogenesis as defined by the number of zygotic embryos displaying somatic embryogenesis after 24 days in culture showed highly significant Pearson's correlation (P<0.0001) with late callus diameter, late somatic embryogenesis, and number of plantlets regenerated with correlations at 0.8457, .8980, and 0.5948, respectively (Table 4.5). Early callus diameter compared to late callus diameter showed a correlation of 0.7736 (P<0.0001). Since the number of zygotic embryos displaying callus with somatic embryos had a higher correlation 0.8980, simple count data could suffice in determining genotypes with efficient tissue culture response in lieu of measuring callus diameter. However, significant marker-trait associations spanning this QTL region suggest that the region could control more than one tissue culture trait metric, and therefore, including more phenotypic trait measurements could aid in parsing out sources of phenotypic variation in tissue culture response.

Multiple marker-trait associations are significantly associated with embryogenic, regenerable tissue culture response.

The six flanking SNP markers used in this study to differentiate between segments spanning across the QTL region on chromosome 3 were all found to be statistically significantly associated with early and late callus diameter and somatic embryogenesis ratings ranging with levels of significance from (P<0.0001) to (P< 0.04) (Table 4.9). These results suggest that each significant marker-trait association could represent fractionation. Fractionation is the phenomenon that a major QTL could fractionate, or are actually multiple tightly linked genes co-localized in the same region [22,23]. In addition, the two highly significant markers could be displaying pleiotropy, where potentially one major QTL underlies a single gene with multiple phenotypic effects. Fractionation is common in high resolution mapping and also an indication of complex trait architecture [23,24]. Two markers in particular, PZE-103122471 and SYN29001 were highly significant for all four tissue culture traits (P<0.0001). The physical distance between markers is 3,053kb or a genetic distance of 4 cM as determined by SNP genotyping of 2,243 recombinants to determine the recombination frequency in this interval (Table 4.2).

Candidate genes in the QTL region are associated with tissue culture related gene functions

Based on the current maize B73 reference genome filtered gene set, there are an estimated 94 genes in the interval between these two SNP. Some of these genes are related to hormone metabolism such as abscisic acid induced regulators, auxin-induced regulators and brassinosteriod synthesis-degradation related genes. Some genes are also described as transcription factors such as ZmWOX2A (GRMZM2G108933) which is a Homeobox transcription factors. GRMZM2G157679 is a GRAS transcription factor highly similar to

SCARECROW involved in gibberellin response. Other genes that could also be related to embryogenesis or tissue culture response efficiency are stress related genes such as AC209784.3_FG007 which is a heat shock protein, and GRMZM2G306258 and GRMZM2G349651 which are chromatin-related histone H2E and H4 genes, respectively.

4.5. CONCLUSION

This study sought to increase the resolution in the genetic and physical map of a QTL region associated with embryogenic, regenerable tissue culture response in a near-isogenic, doubled haploid line using SNP markers to associate specific traits to chromosomal regions, with the long-term goal to identify candidate genes by map-based cloning. Two flanking SNP markers, PZE-103122471 and SYN29001 that span a 3,053 kb interval on chromosome 3 show very high statistical significance to explain the phenotypic variation in five tissue culture response traits: early callus diameter, early somatic embryogenesis, late callus diameter and late somatic embryogenesis, and plantlet regeneration response. This finding suggests that a major gene or genes controlling high embryogenic, regenerable tissue culture response could be identified in this region. High resolution mapping analysis showed that the test of the effect of the QTL could be displaying fractionation or pleiotropy. Future studies focusing on a higher SNP density, more phenotypes, and more entries and replicates should aid in reaching the ultimate goal of understanding the genetic mechanisms that control somatic embryogenesis and efficient response in tissue culture in maize and other crops.

Table 4.1. Single nucleotide polymorphic markers on maize chromosome 3 flanking segments of a near-isogenic line, DH99, used to screen F_2 recombinant seed chips for population development in fine-mapping for candidate genes in a putative quantitative trait loci region

SNP ID	Sequence
PZE-103105125	TGGCAGTTCGATCTTAATAACTTCAAAGACTCCCTTTGTTACTGAAGTTA[A/G]TTGCACAGTTCATATTATCTTATTCTACTATGCAGGTTGCTGCGCTTGTT
PZE-103107449	GCAATCACAACAGACGATGCCGAACCTGGAATCACCCAAACAGAGTCCAT[A/G]GTGTGATTTTCATCCGCGACAAGCCGGACCCTTTTCTTCTAGATTGTTTT
PZE-103122471	CCGCCACCTTATCCACCAAAGGCAACAGAACAGATTTTAGTTGGTTTCTT[A/G]ATGGGGAGAGGAAGGCCAAGATAAGTGCACGGAAAGCTGCTGACAGCACA
SYN29001	CTCCCATGCTGTTGTTGACTTATTGCACAATTCTTCTTCTTCCCAATGAGCGTCAACCCC[A/G]AGTCGAGCAATGCTCTCGTCAATGAAGCTGCGGCGTGCAGAGGCGTAGGTCTCCGCACAC
PZE-103133772	CAATGATCTTGCTGGCAGCCTGGCACTCGGAGTCGGAGTAGGTGTGAAGC[A/G]ACAGTAGTAATCTATTCGAACCACAGCATGCACTTGGACAGTCCCGTGTA
PZE-103135061	ACAGTATGCAGTATCCAGTGTGGTTTCAGAATTCAGAATCCAAGTGACAC[A/C]TTTCTGTGATGAGATGGCCGATTTTTTACCGACAAAATTTGGCGTGCTGC

Table 4.2. Genetic mapping information as intervals described by flanking six single nucleotide polymorphic markers (SNP) to describe the recombination frequency and centimorgan (cM) distance between the markers that were used to genotype 2,243 segregating F_2 recombinant single seeds from a fine-mapping population between maize lines B73 X DH99

			Interval				
Segment	SNP	Base pairs	EF	FG	GH	HI	IJ
E	PZE-103105125	164,821,641	2cM				
F	PZE-103107449	166,794,453		9cM			
G	PZE-103122471	178,772,856			4cM		
H	SYN29001	181,826,658				5cM	
I	PZE-103133772	187,789,641					1cM
J	PZE-103135061	188,705,744					
		Number of recombinants:	32	170	75	98	19
		Recombination Frequency	2%	9%	4%	5%	1%
		Estimated number of genes	45	269	94	166	27

Table 4.3 Number of replicates per homozygous recombinant F_3 source seeds categorized by genotypic type tested in a fine-mapping experiment for tissue culture traits in a cross between B73 X DH99

Genotypic Type	Source ear	Number of replicates
A	WIPV13/000200	3
	WIPV13/000201	8
	WIPV13/000202	6
	Total	17
B	WIPV13/000022	2
	WIPV13/000026	1
	WIPV13/000028	3
	WIPV13/000049	1
	WIPV13/000050	1
	WIPV13/000051	1
	WIPV13/000052	3
	WIPV13/000058	5
	WIPV13/000062	2
	WIPV13/000064	1
	WIPV13/000127	2
	WIPV13/000128	1
	Total	23
C	WIPV13/000058	1
	WIPV13/000134	1
	Total	2
D	WIPV13/000143	2
	Total	2
E	WIPV13/000002	2
	WIPV13/000004	2
	WIPV13/000027	2
	WIPV13/000084	1
	WIPV13/000104	1
	WIPV13/000107	1
	Total	9
F	WIPV13/000037	2
	Total	2
G	WIPV13/000027	5
	WIPV13/000031	2
	WIPV13/000043	2
	WIPV13/000047	1
	WIPV13/000048	4
	WIPV13/000066	1
	WIPV13/000123	2
	WIPV13/000126	1
	WIPV13/000145	2
	Total	20

H	WIPV13/000015	1
	WIPV13/000124	3
	Total	4

Table 4.3 continued

Genotypic Type	Source ear	Number of replicates
I	WIPV13/000035	1
	Total	1
J	WIPV13/000094	1
	Total	1
K	WIPV13/000040	2
	WIPV13/000057	2
	Total	4
L	WIPV13/000022	2
	WIPV13/000031	1
	WIPV13/000075	1
	WIPV13/000087	2
	WIPV13/000114	1
	WIPV13/000122	1
	WIPV13/000135	2
	Total	10
M	WIPV13/000020	4
	WIPV13/000026	1
	WIPV13/000028	2
	WIPV13/000037	2
	WIPV13/000054	1
	WIPV13/000109	3
	WIPV13/000110	3
	WIPV13/000115	1
	WIPV13/000143	1
	Total	18
N	WIPV13/000138	1
	WIPV13/000145	2
	WIPV13/000147	2
	Total	5
O	WIPV13/000203	11
	Total	11
	Grand Total	129

Table 4.4. Early and late tissue culture traits counted and measured in F4 embryos in tissue culture in a fine-mapping experiment in a cross between B73 X DH99 including agronomic traits on greenhouse plants

Phenotype	N	Mean	Standard Deviation	Minimum	Maximum
Tissue culture traits					
Early callus diameter	128	0.47	0.22	0.24	1.47
Early SE/ZE	128	15.37	15.85	0	50
Late callus diameter	129	1.05	0.79	0.32	3.53
Late SE/ZE	129	6.05	7.88	0	20
PRL	129	12.31	21.91	0	103
Agronomic traits					
Ear height	128	97	18.87	49	140
Plant height	128	200	24.62	112	249
Number of ears and shoots	128	3	0.97	1	6
Number of tillers	128	1	1.09	0	4

Table 4.5. Pearson correlation coefficients, p-values, and the number of entries tested in a fine-mapping experiment for tissue culture traits in maize where early measurements were taken 24 days after plating, late measurements were taken 38 days after plating, and the total number of plantlets regenerated was taken 76 days after plating in tissue culture.

	Early callus diameter	Early somatic embryogenesis	Late callus diameter	Late somatic embryogenesis	Plantlets regenerated
Early callus diameter	1	0.6708	0.7736	0.6867	0.2205
		<.0001***	<.0001***	<.0001***	0.012*
	129	129	129	129	129
Early somatic embryogenesis	0.6708	1	0.8457	0.8980	0.5948
	<.0001***		<.0001***	<.0001***	<.0001***
	129	129	129	129	129
Late callus diameter	0.7736	0.8457	1	0.9370	0.5794
	<.0001***	<.0001***		<.0001***	<.0001***
	129	129	129	129	129
Late somatic embryogenesis	0.6867	0.8980	0.9370	1	0.7234
	<.0001***	<.0001***	<.0001***		<.0001***
	129	129	129	129	129
Plantlets regenerated	0.2205	0.5948	0.5794	0.7234	1
	0.012*	<.0001***	<.0001***	<.0001***	
	129	129	129	129	129

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 4.6. Statistical tests for normality and goodness of fit tests in a fine-mapping experiment in maize tissue culture traits

	Early callus diameter				Early somatic embryogenesis			
Test	Statistic		p Value		Statistic		p Value	
Shapiro-Wilk	W	0.74	Pr < W	<0.0001***	W	0.84	Pr < W	<0.0001***
Kolmogorov-Smirnov	D	0.25	Pr > D	<0.0100**	D	0.18	Pr > D	<0.0100**
Cramer-von Mises	W-Sq	2.16	Pr > W-Sq	<0.0050**	W-Sq	1.22	Pr > W-Sq	<0.0050**
Anderson-Darling	A-Sq	11.80	Pr > A-Sq	<0.0050**	A-Sq	7.35	Pr > A-Sq	<0.0050**
	Late callus diameter				Late somatic embryogenesis			
Test	Statistic		p Value		Statistic		p Value	
Shapiro-Wilk	W	0.79	Pr < W	<0.0001***	W	0.73	Pr < W	<0.0001***
Kolmogorov-Smirnov	D	0.26	Pr > D	<0.0100**	D	0.29	Pr > D	<0.0100**
Cramer-von Mises	W-Sq	2.08	Pr > W-Sq	<0.0050**	W-Sq	2.60	Pr > W-Sq	<0.0050**
Anderson-Darling	A-Sq	11.14	Pr > A-Sq	<0.0050**	A-Sq	15.12	Pr > A-Sq	<0.0050**

Plantlet regeneration response				
Test	Statistic		p Value	
Shapiro-Wilk	W	0.64	Pr < W	<0.0001***
Kolmogorov-Smirnov	D	0.34	Pr > D	<0.0100**
Cramer-von Mises	W-Sq	3.84	Pr > W-Sq	<0.0050**
Anderson-Darling	A-Sq	19.5	Pr > A-Sq	<0.0050**

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 4.7. Analysis of variance of in a fine-mapping experiment in maize for tissue culture trait data

Early callus diameter					
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	49	4.15	0.08	2.74	<.0001***
Error	78	2.41	0.03		
Corrected Total	127	6.57			
R-Square	Coeff Var	Root MSE	E_diam Mean		
0.63	37.62	0.18	0.47		
Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	1	0.07	0.07	2.42	0.1242
gh	2	0.03	0.01	0.46	0.6317
rep(gh)	3	0.09	0.03	0.94	0.4238
geno	14	1.60	0.11	3.69	<.0001***
time*gh	1	0.01	0.01	0.33	0.5691
time*geno	8	0.23	0.03	0.92	0.5014
gh*geno	14	0.36	0.03	0.83	0.6305
time*gh*geno	4	0.21	0.05	1.71	0.1572
Early somatic embryogenesis					
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	49	21056.98	429.73	3.09	<.0001***
Error	78	10862.49	139.26		
Corrected Total	127	31919.47			
R-Square	Coeff Var	Root MSE	E_SE Mean		
0.66	76.83	11.80	15.36		
Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	1	510.92	510.92	3.67	0.0591
gh	2	260.18	130.09	0.93	0.3973
rep(gh)	3	352.87	117.62	0.84	0.4736
geno	14	11706.69	836.19	6.00	<.0001***
time*gh	1	39.42	39.42	0.28	0.5962
time*geno	8	861.86	107.73	0.77	0.6270
gh*geno	14	2393.66	170.98	1.23	0.2731
time*gh*geno	4	570.34	142.59	1.02	0.4003

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 4.7. continued

Late callus diameter

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	49	59.23	1.21	4.63	<.0001***
Error	78	20.35	0.26		
Corrected Total	127	79.58			

R-Square	Coeff Var	Root MSE	L_diam Mean
0.74	48.55	0.51	1.05

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	1	0.00	0.00	0.00	0.9624
gh	2	0.48	0.24	0.92	0.4009
rep(gh)	3	0.42	0.14	0.53	0.6622
geno	14	31.36	2.24	8.59	<.0001***
time*gh	1	0.16	0.16	0.63	0.4306
time*geno	8	2.17	0.27	1.04	0.4136
gh*geno	14	2.77	0.20	0.76	0.7091
time*gh*geno	4	0.64	0.16	0.61	0.6560

Late somatic embryogenesis

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	49	5877.44	119.95	4.59	<.0001***
Error	78	2040.24	26.16		
Corrected Total	127	7917.68			

R-Square	Coeff Var	Root MSE	L_SE Mean
0.74	83.82	5.11	6.10

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	1	35.74	35.74	1.37	0.2460
gh	2	60.26	30.13	1.15	0.3214
rep(gh)	3	82.53	27.51	1.05	0.3745
geno	14	3174.77	226.77	8.67	<.0001***
time*gh	1	41.13	41.13	1.57	0.2136
time*geno	8	252.49	31.56	1.21	0.3063
gh*geno	14	420.53	30.04	1.15	0.3316
time*gh*geno	4	104.81	26.20	1.00	0.4119

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 4.7. continued

Plantlet regeneration response

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	49	32865.61	670.73	1.84	0.0079**
Error	78	28433.27	364.53		
Corrected Total	127	61298.88			

R-Square	Coeff Var	Root MSE	PRL Mean
0.54	153.90	19.09	12.41

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	1	1063.05	1063.05	2.92	0.0917
gh	2	1210.60	605.30	1.66	0.1967
rep(gh)	3	1412.48	470.83	1.29	0.2832
geno	14	16255.26	1161.09	3.19	0.0006***
time*gh	1	1631.73	1631.73	4.48	0.0376
time*geno	8	3341.04	417.63	1.15	0.3429
gh*geno	14	5763.69	411.69	1.13	0.3468
time*gh*geno	4	3725.10	931.28	2.55	0.0453*

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 4.8 Wilcoxon Scores (Rank Sums) by genotypic type (geno) of tissue culture trait data in a fine-mapping experiment in maize

Early somatic embryogenesis						Early callus diameter				
geno	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score	geno	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
A	17	1906.0	1096.5	142.2	112.1	C	227.5	129.0	52.0	113.8
C	2	193.0	129.0	52.0	96.5	A	1885.5	1096.5	142.3	110.9
D	2	183.5	129.0	52.0	91.8	B	1713.5	1419.0	158.1	77.9
K	4	331.5	258.0	72.9	82.9	F	144.0	129.0	52.0	72.0
B	22	1793.0	1419.0	158.1	81.5	E	567.0	580.5	107.2	63.0
F	2	133.0	129.0	52.0	66.5	O	681.0	709.5	117.5	61.9
H	4	251.0	258.0	72.9	62.8	M	963.0	1161.0	145.7	53.5
E	9	528.5	580.5	107.1	58.7	L	503.5	645.0	112.5	50.4
G	20	990.0	1290.0	152.1	49.5	K	193.0	258.0	72.9	48.3
L	10	472.0	645.0	112.4	47.2	D	89.0	129.0	52.0	44.5
O	11	489.5	709.5	117.4	44.5	H	175.5	258.0	72.9	43.9
J	1	43.0	64.5	36.9	43.0	G	865.0	1290.0	152.2	43.3
M	18	746.5	1161.0	145.7	41.5	N	190.0	322.5	81.2	38.0
N	5	167.0	322.5	81.2	33.4	J	34.5	64.5	36.9	34.5
I	1	28.5	64.5	36.9	28.5	I	24.0	64.5	36.9	24.0
Late somatic embryogenesis						Late callus diameter				
geno	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score	geno	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
A	17	1966.0	1105.0	136.0	115.6	C	241.0	130.0	52.4	120.5
C	2	220.0	130.0	49.7	110.0	A	1954.0	1105.0	143.4	114.9
D	2	174.0	130.0	49.7	87.0	B	2048.5	1495.0	162.3	89.1
B	23	1987.5	1495.0	153.9	86.4	D	165.5	130.0	52.4	82.8
F	2	120.5	130.0	49.7	60.3	F	140.5	130.0	52.4	70.3
E	9	501.0	585.0	102.4	55.7	O	574.5	715.0	118.4	52.2
K	4	211.5	260.0	69.7	52.9	H	205.5	260.0	73.5	51.4
H	4	205.0	260.0	69.7	51.3	E	434.0	585.0	108.0	48.2
O	11	559.0	715.0	112.3	50.8	K	192.0	260.0	73.5	48.0
G	20	948.0	1300.0	145.5	47.4	G	953.0	1300.0	153.4	47.7
M	18	840.0	1170.0	139.3	46.7	M	809.5	1170.0	146.9	45.0
L	10	439.0	650.0	107.5	43.9	L	430.5	650.0	113.4	43.1
I	1	30.5	65.0	35.3	30.5	N	201.5	325.0	81.8	40.3
J	1	30.5	65.0	35.3	30.5	I	25.0	65.0	37.2	25.0
N	5	152.5	325.0	77.6	30.5	J	10.0	65.0	37.2	10.0

Table 4.8. continued

Plantlet regeneration response					
Genotype	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	2	224.0	130.0	47.0	112.0
D	2	201.5	130.0	47.0	100.8
A	17	1646.5	1105.0	128.7	96.9
B	23	1996.5	1495.0	145.7	86.8
F	2	146.5	130.0	47.0	73.3
E	9	513.0	585.0	96.9	57.0
N	5	265.5	325.0	73.5	53.1
K	4	212.0	260.0	66.0	53.0
L	10	518.0	650.0	101.8	51.8
G	20	1022.0	1300.0	137.7	51.1
O	11	558.0	715.0	106.3	50.7
H	4	200.0	260.0	66.0	50.0
M	18	805.5	1170.0	131.9	44.8
I	1	38.0	65.0	33.4	38.0
J	1	38.0	65.0	33.4	38.0

Table 4.9. Marker-trait associations for tissue culture traits using raw data for early and late callus diameter and somatic embryogenesis

SNP marker	Level of	Early callus diameter N	Mean	Std Dev	F Value	Pr > F	R^2	Early somatic embryogenesis N	Mean	Std Dev	F Value	Pr > F	R^2
PZE-103105125	A188	58	0.51	0.27	3.39	0.0680	0.03	58	20.03	17.74	9.84	0.0021**	0.07
	B73	70	0.44	0.18				70	11.50	12.99			
PZE-103107449	A188	57	0.52	0.27	6.09	0.0149*	0.05	57	20.35	17.64	10.96	0.0012**	0.08
	B73	71	0.43	0.17				71	11.37	13.05			
PZE-103122471	A188	55	0.59	0.29	38.35	<.0001***	0.23	55	25.13	17.07	50.99	<.0001***	0.29
	B73	73	0.38	0.07				73	8.01	9.84			
SYN29001	A188	62	0.57	0.28	27.03	<.0001***	0.18	62	23.27	17.38	38.87	<.0001***	0.24
	B73	66	0.38	0.07				66	7.94	9.57			
PZE-103133772	A188	75	0.53	0.27	15.55	0.0001	0.11	75	20.11	17.04	18.43	<.0001***	0.13
	B73	53	0.38	0.08				53	8.66	11.04			
PZE-103135061	A188	78	0.52	0.27	13.09	0.0004	0.09	78	18.97	17.22	11.18	0.0011	0.08
	B73	50	0.38	0.08				50	9.74	11.49			

Table 4.9
continued

SNP marker	Level of	Late callus diameter N	Mean	Std Dev	F Value	Pr > F	R^2	Late somatic embryogenesis N	Mean	Std Dev	F Value	Pr > F	R^2
PZE-103105125	A188	58	1.20	0.91	4.05	0.0462*	0.03	58	7.62	8.60	4.27	0.0409*	0.03
	B73	71	0.92	0.66				71	4.77	7.05			
PZE-103107449	A188	57	1.28	0.94	9.33	0.0028**	0.07	57	8.33	8.79	9.07	0.0031**	0.07
	B73	72	0.86	0.60				72	4.25	6.60			
PZE-103122471	A188	56	1.62	0.87	88.00	<.0001***	0.41	56	11.73	8.25	85.04	<.0001***	0.40
	B73	73	0.61	0.29				73	1.70	3.76			
SYN29001	A188	63	1.51	0.88	60.81	<.0001***	0.32	63	10.71	8.56	64.31	<.0001***	0.34
	B73	66	0.61	0.29				66	1.61	3.36			
PZE-103133772	A188	76	1.32	0.88	26.89	<.0001***	0.17	76	8.71	8.56	24.93	<.0001***	0.16
	B73	53	0.65	0.40				53	2.25	4.72			
PZE-103135061	A188	79	1.27	0.88	19.21	<.0001***	0.13	79	8.19	8.65	16.81	<.0001***	0.12
	B73	50	0.69	0.44				50	2.68	4.93			

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

Table 4.9 continued

Plantlets regeneration response

SNP marker	Level of	N	Mean	Std Dev	F Value	Pr > F	R^2
PZE-103105125	A188	58	16.74	27.44	4.43 0.0374	0.0374*	3.4%
	B73	71	8.69	15.33			
PZE-103107449	A188	57	18.21	27.65	7.80 0.0060	0.006**	5.8%
	B73	72	7.64	14.58			
PZE-103122471	A188	73	3.66	11.87	32.70	<.0001***	20.5%
	B73	56	23.59	26.53			
SYN29001	A188	66	3.42	10.72	26.69	<.0001***	17.4%
	B73	63	21.62	26.43			
PZE-103133772	A188	76	16.45	23.98	6.90	0.0097**	5.2%
	B73	53	6.38	17.08			
PZE-103135061	A188	50	6.86	17.33	5.22	0.0240*	4.0%
	B73	79	15.76	23.83			

*Significant at $P \leq 0.05$, **Significant at $P \leq 0.01$, ***Significant at $P \leq 0.001$

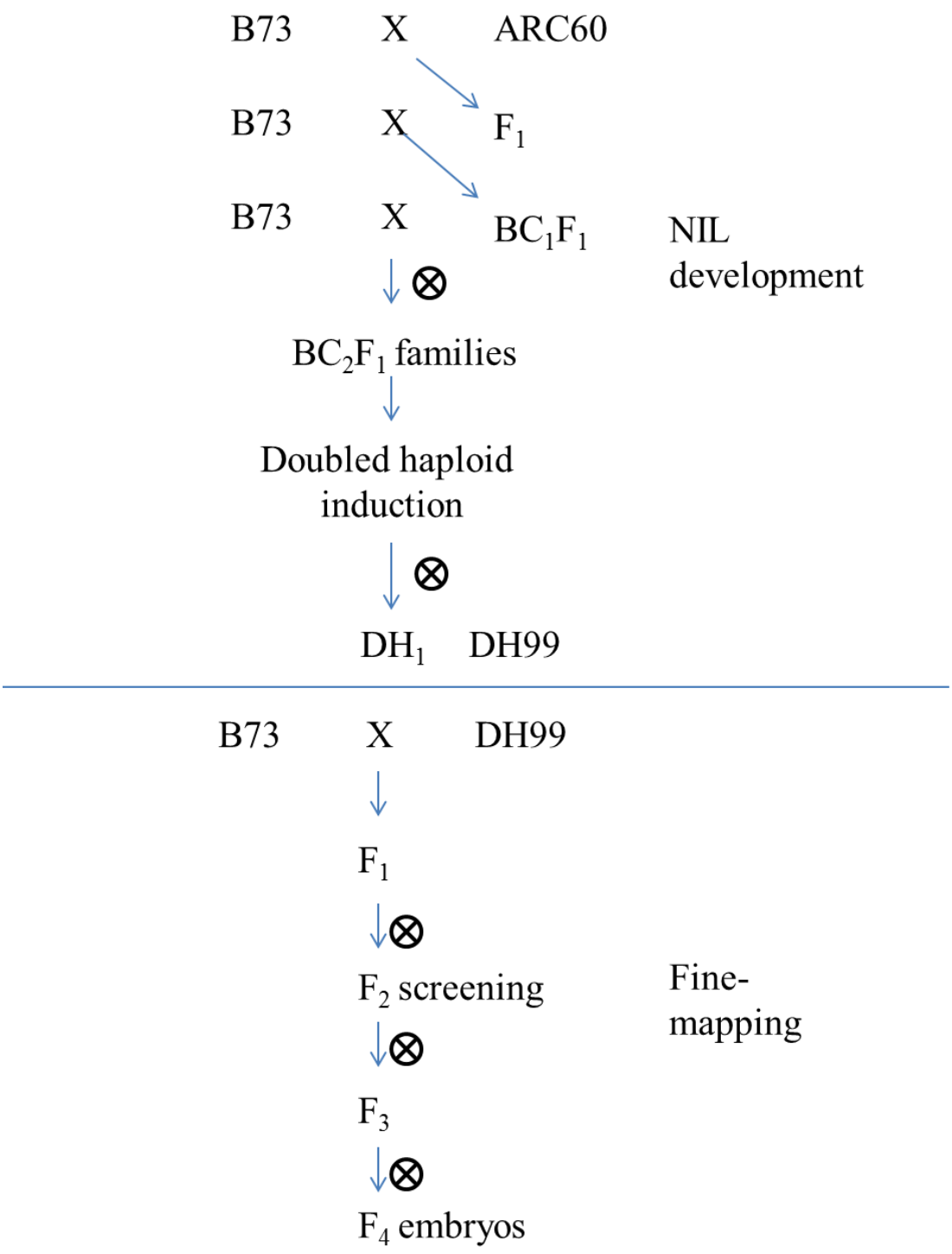

Figure 4.1. Line development of the near-isogenic maize line DH99 efficient in tissue culture response and then utilized for fine-mapping tissue culture traits in maize

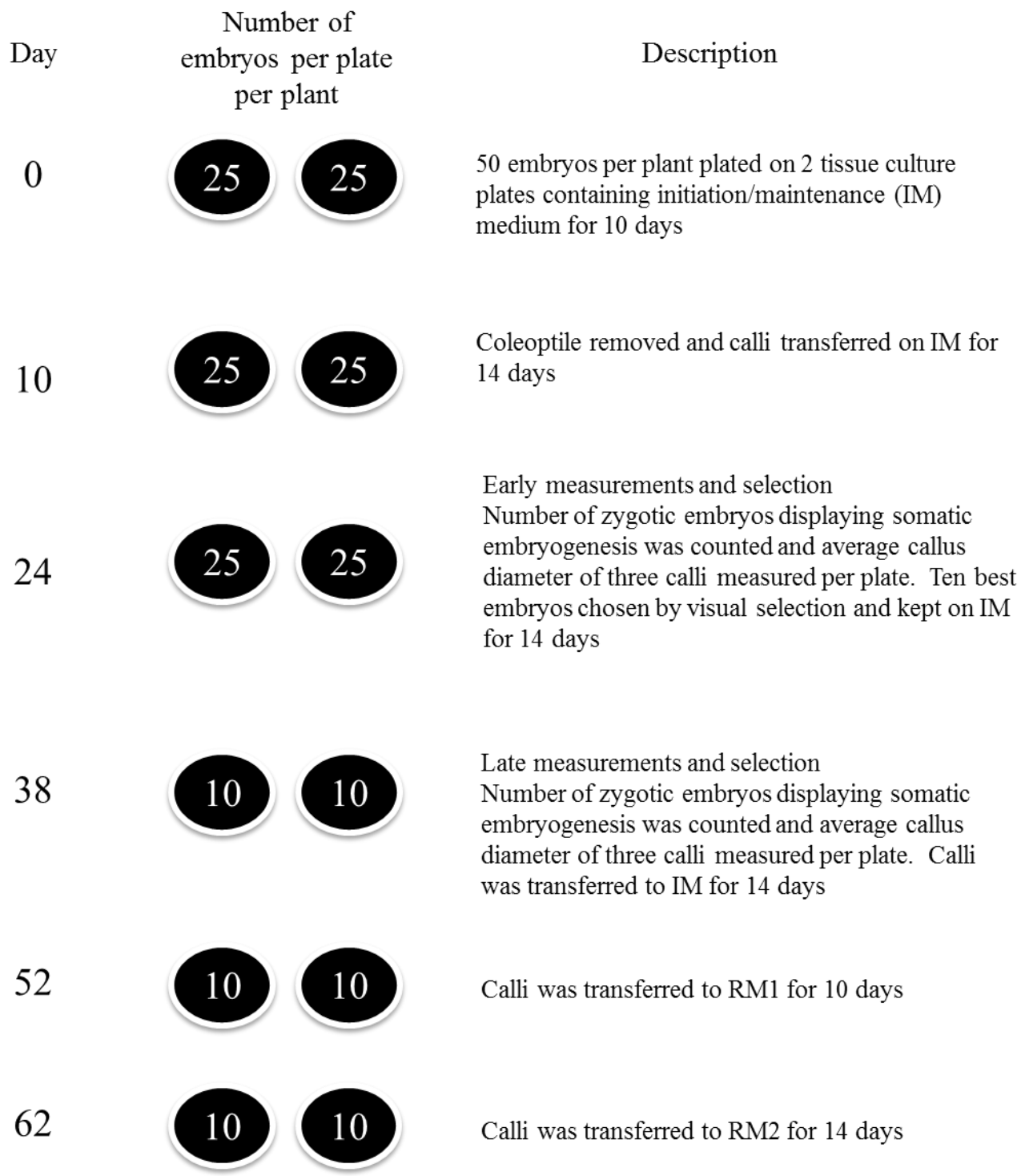

Figure 4.2. Tissue culture response based on callus growth rate based on early and late measurements on callus cultures displaying somatic embryogenesis

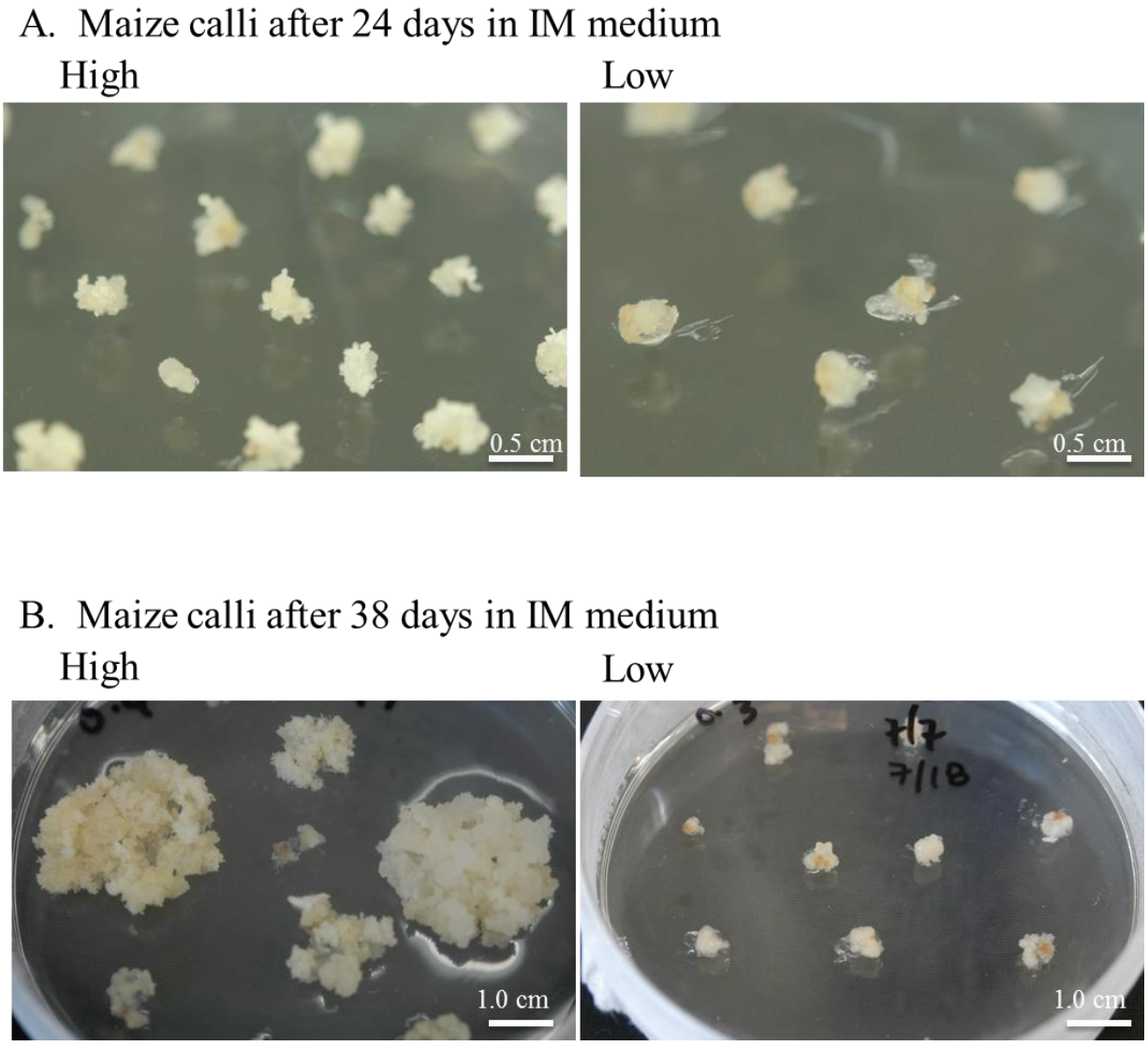

Figure 4.3. Maize calli at (A) 24 or (B) 38 days in initiation/maintenance (IM) medium displaying high and low embryogenic response visible by embryogenic structures and differences in callus growth rate estimated by an increase in callus diameter

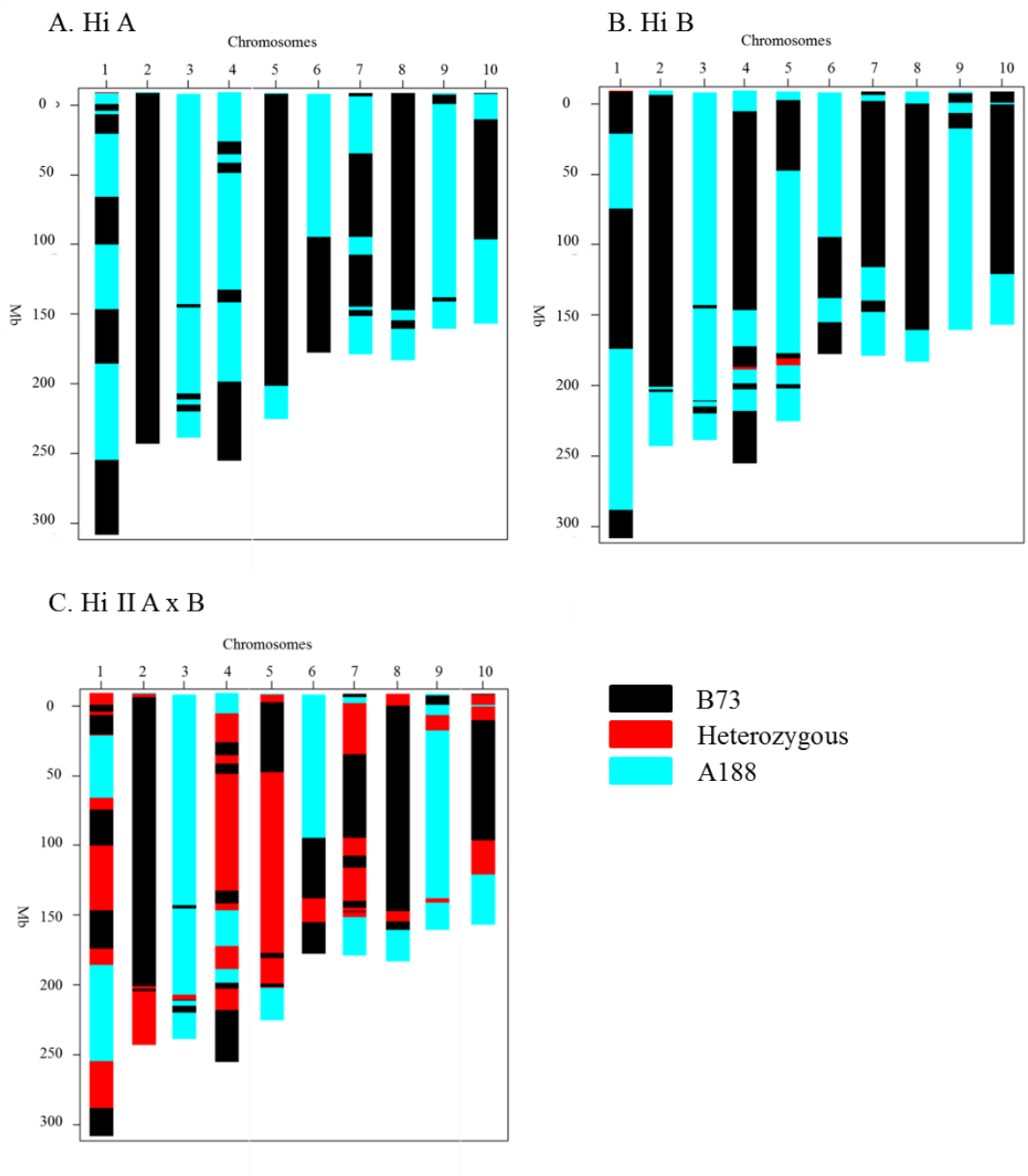

Figure 4.4 Karyotype of all ten chromosomes of the maize (A) Hi A parent, (B) Hi B parent and, (C) the F_1 Hi II A X B hybrid using 55,000 single nucleotide polymorphic markers where turquoise segments are A188 alleles, black segments are B73, and red segments are heterozygous alleles

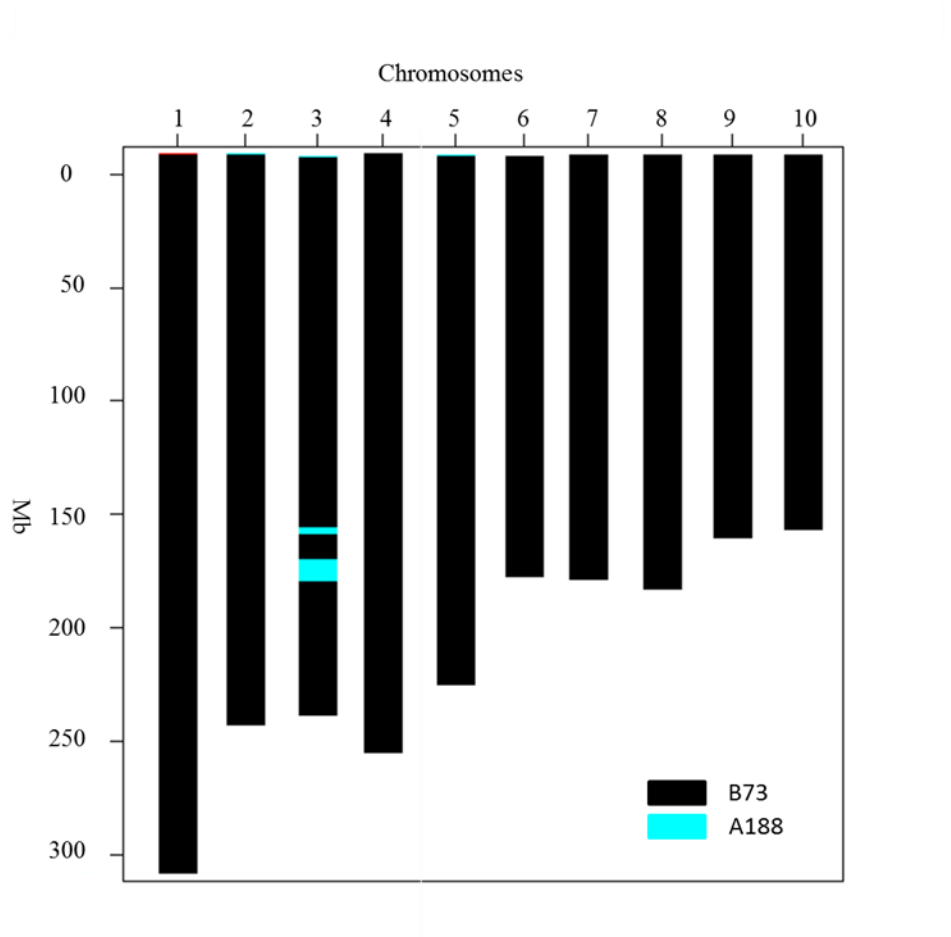

Figure 4.5 Karyotype of all ten chromosomes of the maize near-isogenic line DH99 that harbors two small segments of A188 introgressions genotyped using 55,000 genome-wide single nucleotide polymorphisms (SNPs)

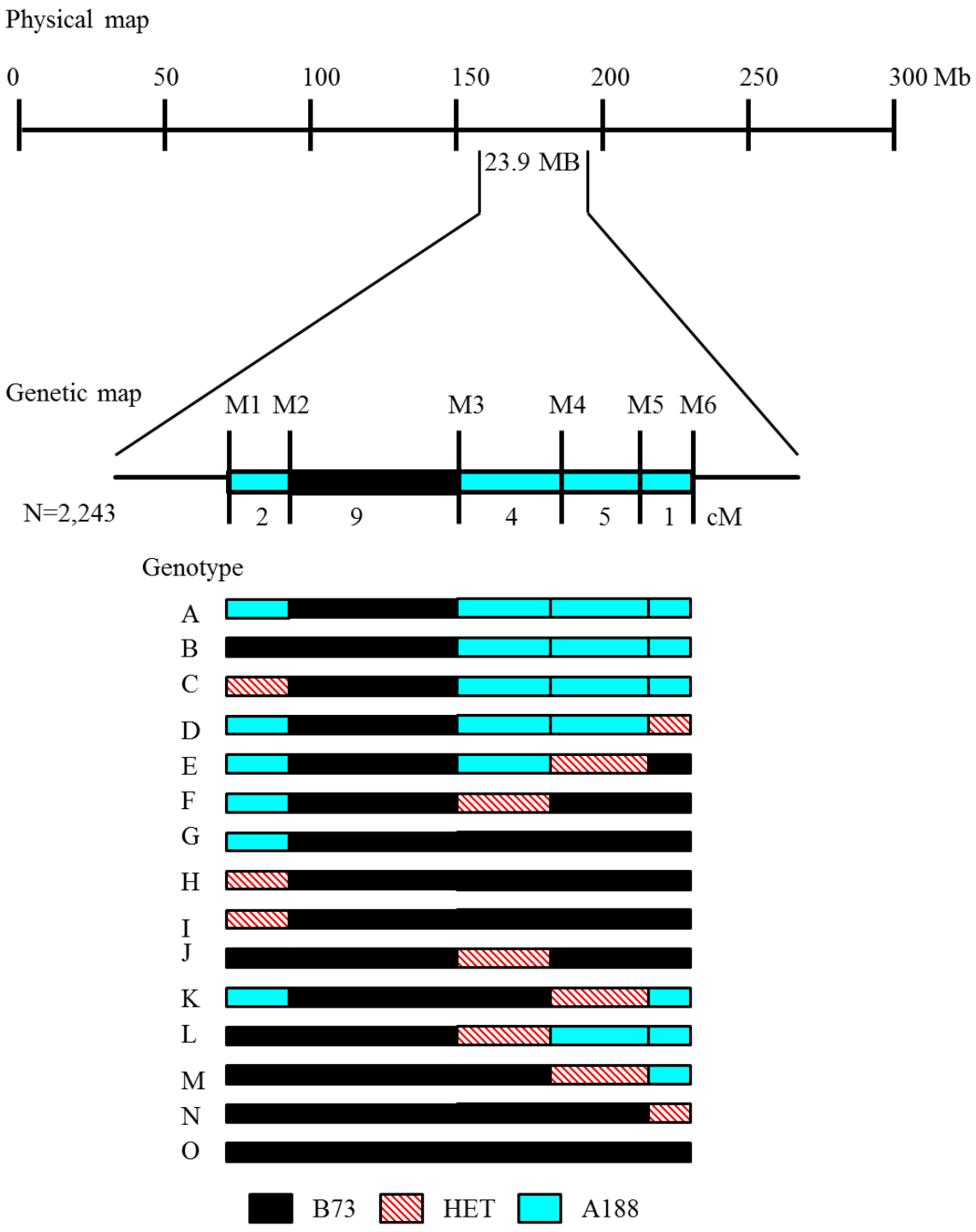

Figure 4.6 Fifteen different genotypic types representing F_3 homozygous recombinant plants tested in a fine-mapping experiment to test for the effect of marker-trait associations for tissue culture response with six SNP markers on maize chromosome 3

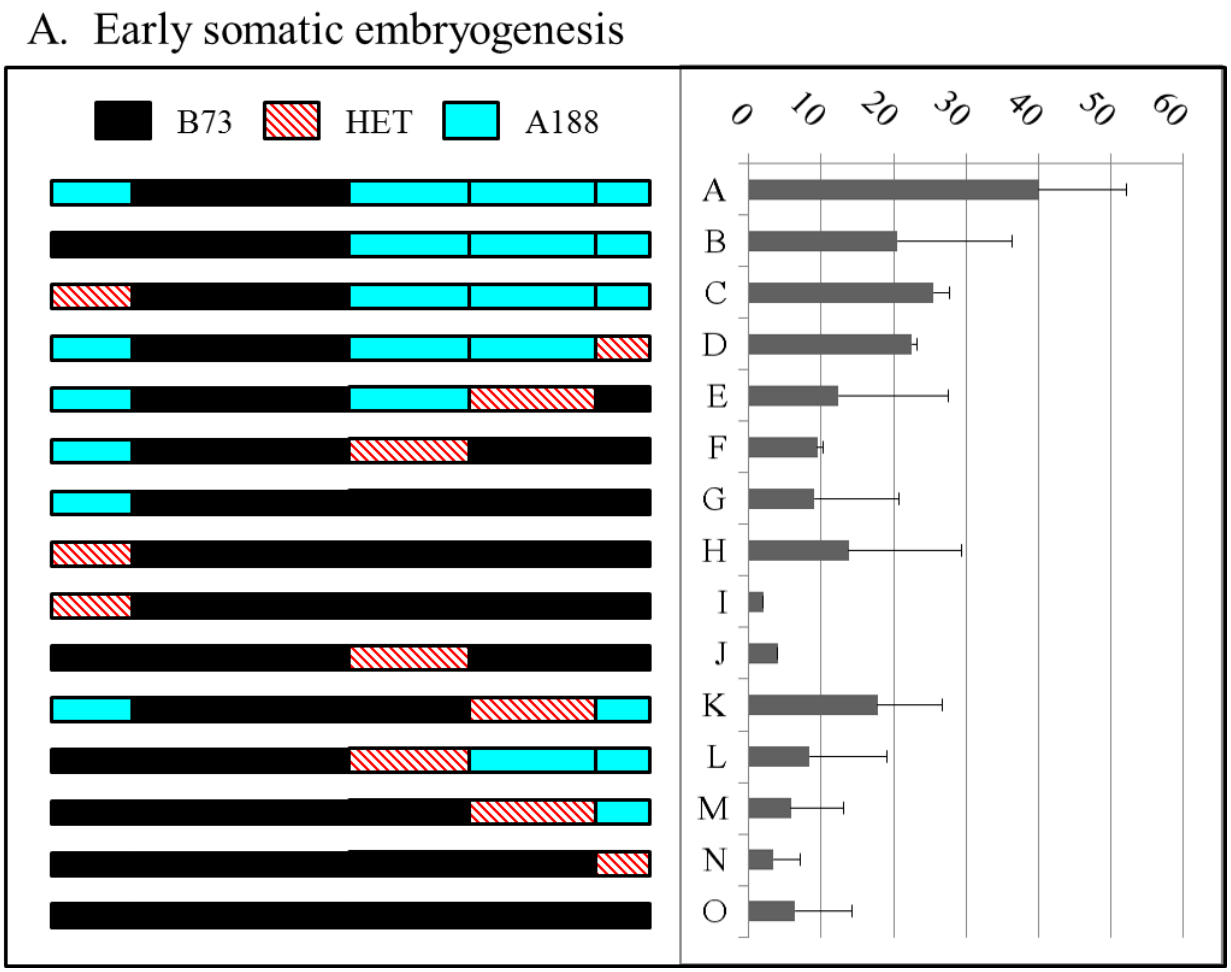

Figure 4.7. Maize tissue culture traits used in fine mapping for embryogenic tissue culture response as (A) the number of zygotic embryos displaying somatic embryogenesis in tissue culture taken 24 days after plating in onto tissue medium (B) the average callus diameter (cm) measured 24 days after plating in tissue culture (C) the number of zygotic embryos displaying somatic embryogenesis in tissue culture taken 38 days after plating in onto tissue medium (D) the average callus diameter (cm) measured 38 days after plating in tissue culture (E) the total number of plantlets regenerated per plant

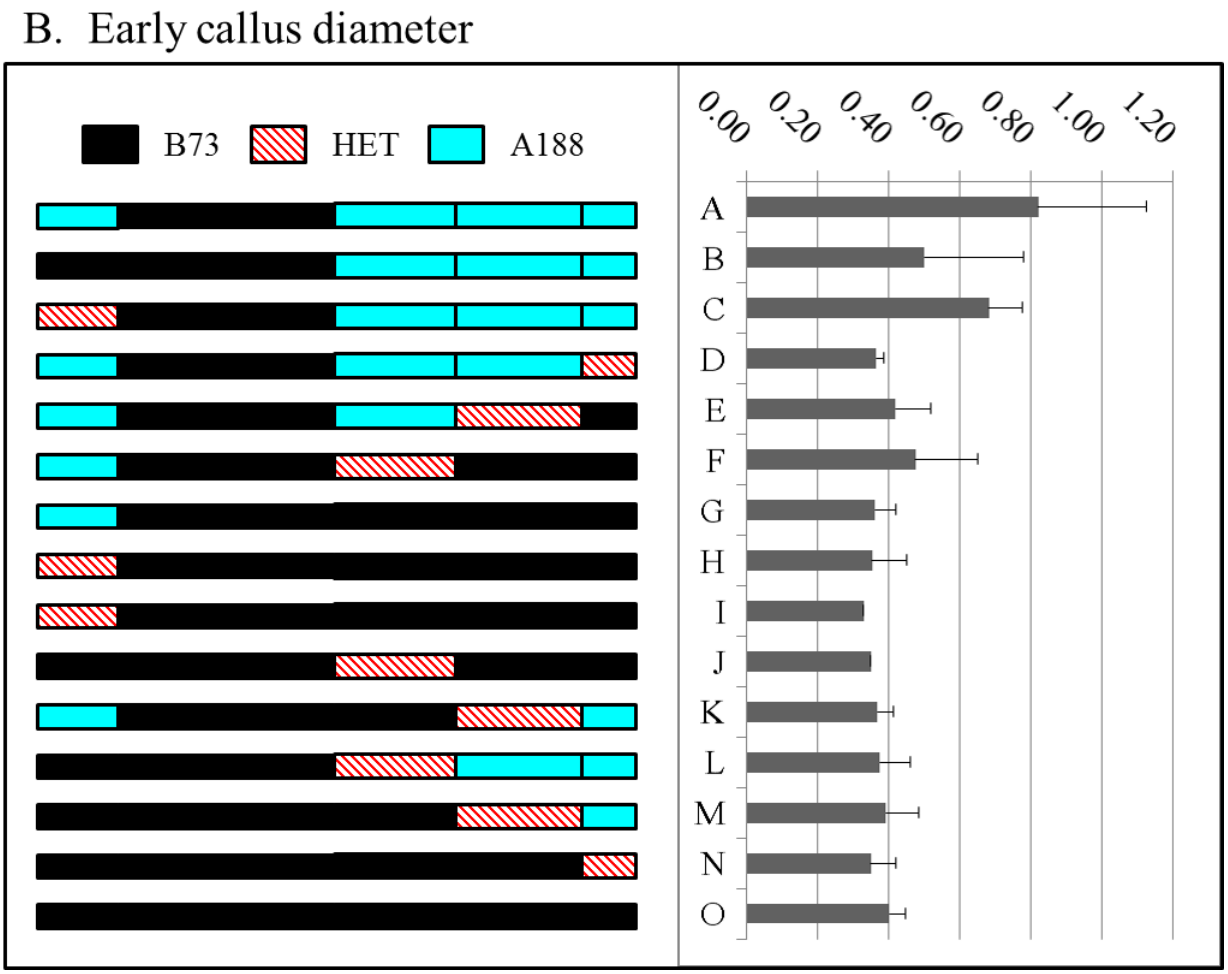

Figure 4.7 continued.

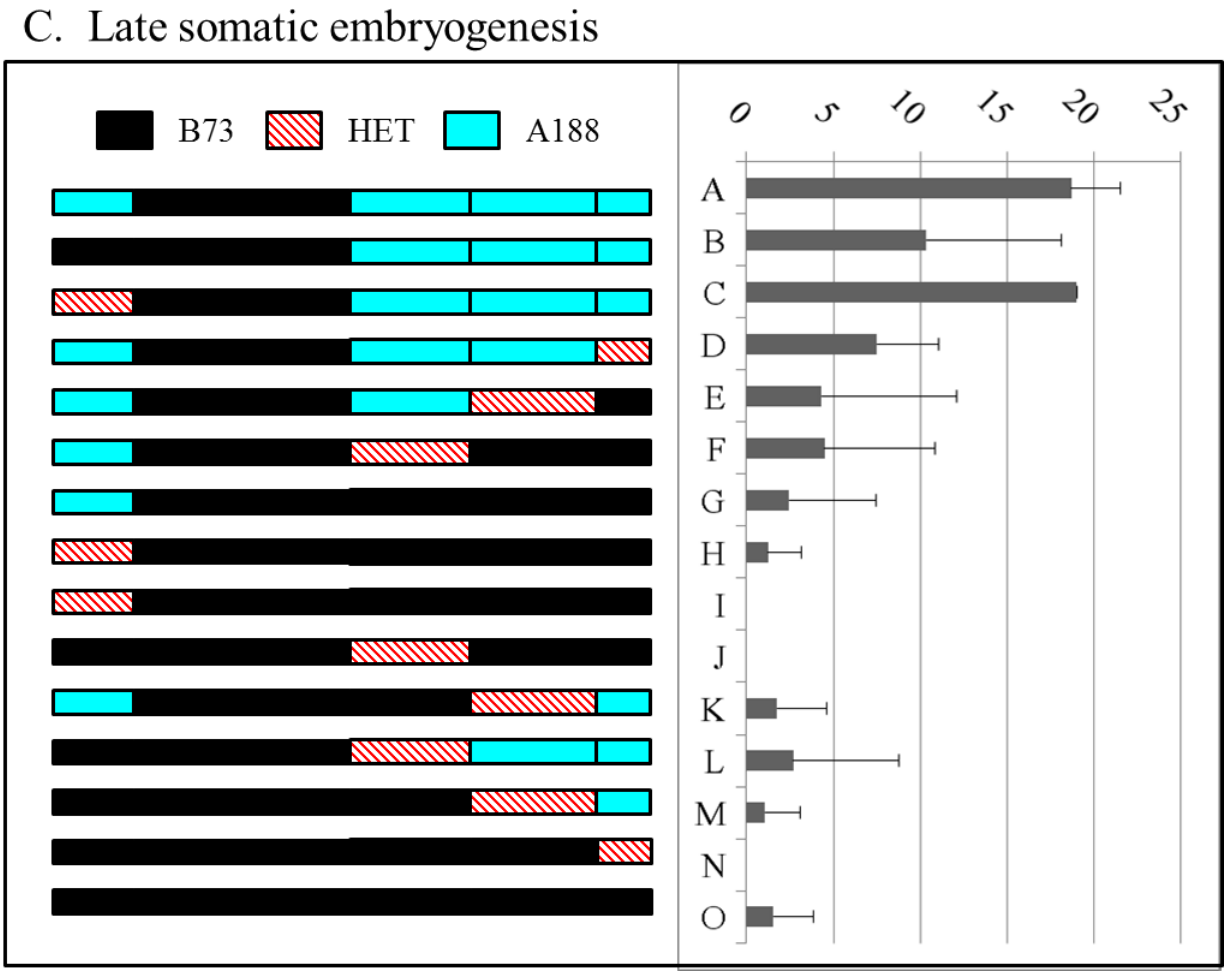

Figure 4.7 continued.

D. Late callus diameter

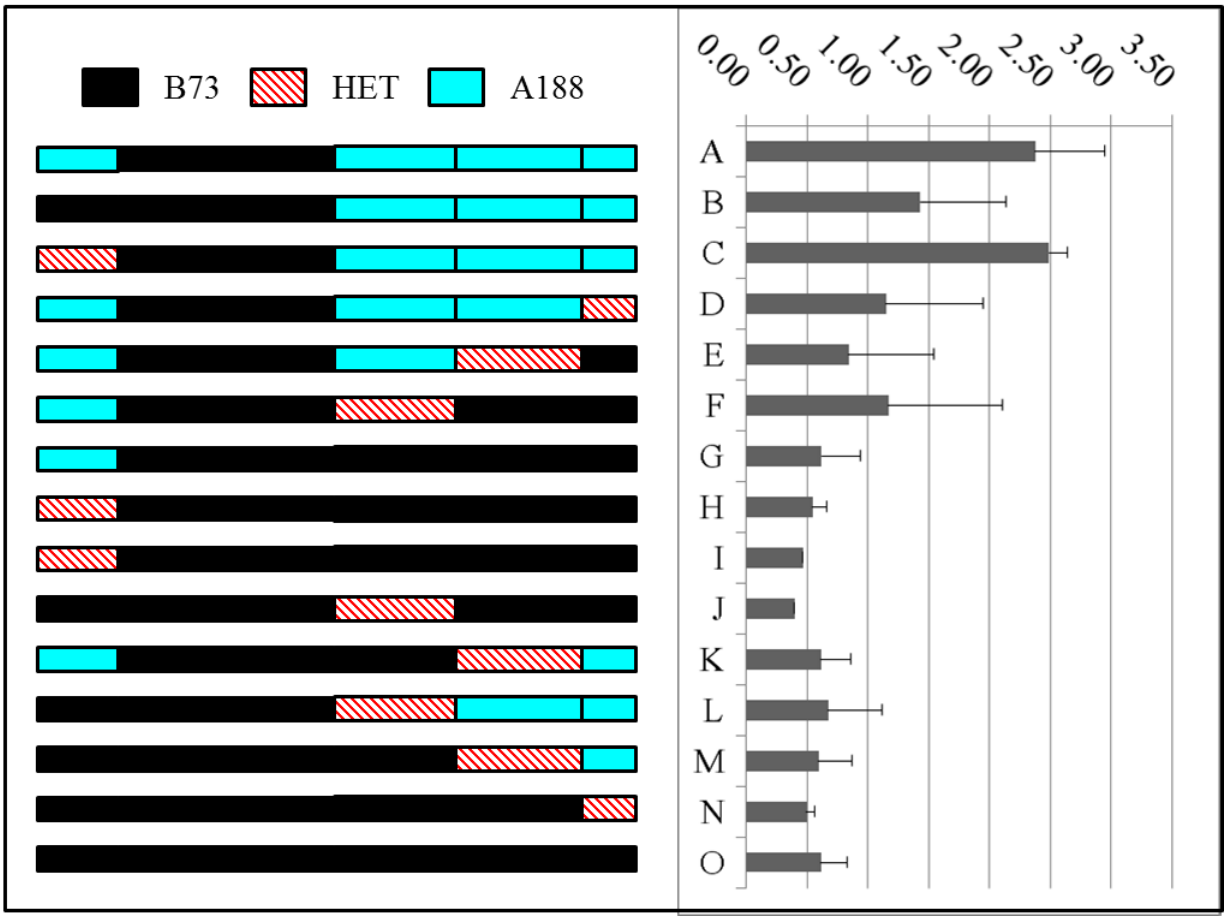

Figure 4.7 continued.

E. Number of plantlets regenerated

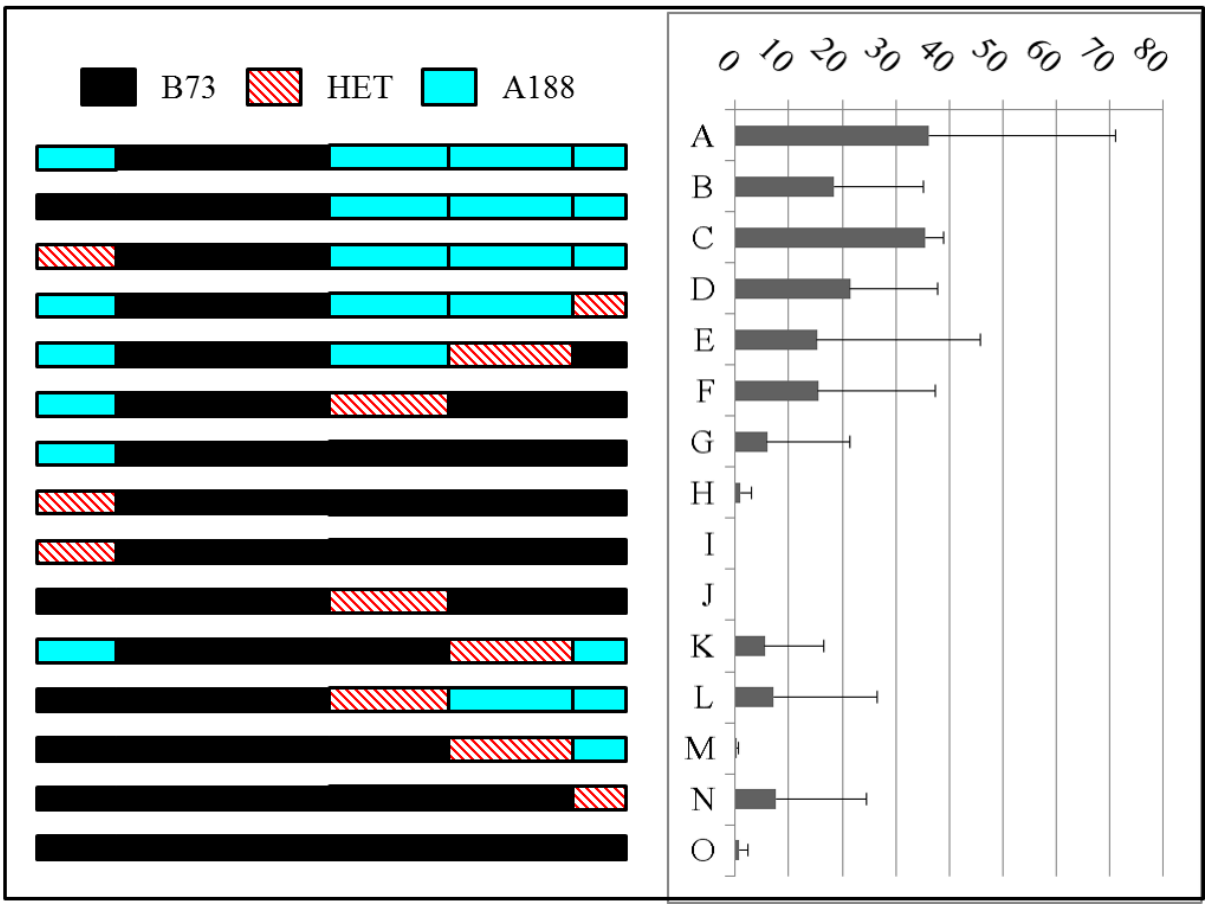

Figure 4.7 (E)

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APPENDICIES

APPENDIX A.1. Protocol for 96-well CTAB extraction (Quickpreps)

Materials necessary:
Riplate 96-well 1.2 mL polypropylene plate(s)
Qiagen tungsten carbide beads (P# 69997)
CTAB buffer base
beta-mercaptoethanol
Qiagen 8-well cap strips (Collection Microtube caps, P# 19566)
tape seal
24:1 chloroform:octyl alcohol
96-well 500 uL polypropylene plate(s) (FisherBrand)
isopropanol
70% ethanol
4 racks of P200 tips. Also, a box of Matrix pipettor tips.

Preliminary steps:

1)Prepare CTAB buffer base on preceding evening.

For 50ml CTAB:In bottle on a stir plate. 25ml H2O, add 1g CTAB and 0.5g Na Bisulfite.

Add 14ml NaCl (5M)
Add 5ml 1M Tris-HCl (pH8.0) Filter sterilized
Add 2ml 0.5M EDTA (pH8.0) Filter sterilized
Let stir until the CTAB and Na Bisulfite is dissolved entirely.
Adjust volume to 50ml and remove stir bar if you want.
Store in fridge and right before use add 500ul beta-mercaptoethanol (1%).

2) Put a 1-square-centimeter leaf cutting into each well of the plate and 1 tungsten carbide bead. Ensure that a well is kept clear for an extraction blank.

On day of extraction:

-preheat oven to 65 degrees
-Add BME to the CTAB base to 1% final concentration.

1) Using electronic multichannel, add 230 uL of the now-complete CTAB buffer to each well. Seal with cap strips, taking care that all caps are fully seated.
2) Shake 30 seconds at 30 Hz in Mixer Mill. Tear down, reseat all caps, set back up. Repeat until 4 total cycles (or more until tissue is broken down) are completed.
3) Centrifuge plates 10 minutes at 4000xg. Remove cap strips and seal with tape.
4) Incubate 30 minutes at 65 degrees Celsius.
5) Remove from oven, add 230 uL chloroform:octanol. Mix by pipetting with wide bore tips (150ul volume) 20x. Seal with tape strip.
6) Centrifuge 10 min at 4000xg.

7) Carefully remove 100 uL of aqueous phase by normal multichannel using “spacers” and transfer to a 500 uL plate.
8) Add 100 uL ice-cold isopropanol to the aqueous phase samples to precipitate. Pipet 10 times to mix (120ul volume). Let stand at room temperature for 10 minutes.
9) Centrifuge plate 10 min at 4000xg to pellet DNA.
10) Carefully remove isopropanol with multichannel pipettor (220ul volume), avoiding pellet. This is most easily done on a dark, level background while tipping plate ~20 degrees. Tap to find wells still with excess isopropanol. Pipet off. Speed vac 5 min (heater off) until appropriately dried.
11) Add 50 uL of 1x TE buffer to each well, Seal with tape seal and put on orbital shaker 75rpm for 1m. Place in refrigerator overnight to resuspend.
To Clean Beads: rinse immediately with isopropanol, rinse with water, do an acid rinse with HCl, rinse well with water, rinse with 95% EtOH and allow to air dry

APPENDIX A.2. DNA Isolation and Purification from Nathan Springer Lab

Tris-Cl (1 M)
Dissolve 121.1 g of Tris base in 800 ml of H2O. Adjust the pH to the desired value by adding concentrated HCl <!>.
pH HCl
7.4 70 ml
7.6 60 ml
8.0 42 ml ßour most common
Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with H2O. Dispense into aliquots and sterilize by autoclaving.
If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature dependant, and decreases ~0.03 pH units for each 1 C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5 C, 25 C, and 37 C, respectively.

NaCl (Sodium Chloride, 5 M)
Dissolve 292 g of NaCl in 800 ml of H2O. Adjust the volume to 1 liter with H2O. Dispense into aliquots and sterilize by autoclaving. Store the NaCl solution at room temperature.

EDTA (0.5 M, pH 8.0)
Add 186.1 g of disodium EDTA*2H2O to 800 ml of H2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

CTAB Extraction Buffer

Final Con.	**Stock Conc**	**Amt of stock to add**
0.1 M Tris pH 7.5	1 M Tris pH 7.5	100ml
0.75M NaCl	5 M NaCl	140ml
0.01M EDTA	0.5M EDTA	20ml
1% CTAB	CTAB	10g
ddH2O		730ml
		1000ml
1% B-mercaptoethanol	14 M BME	10 ml/1000 ml CTAB Buffer (add just before use)

CTAB =1% mixed alkyl trimethyl-ammonium bromide

DNA Wash Solution

76% ethanol _____ 304 ml of 100% ethanol
10 mM NH4Ac _____ 0.4 ml of 10 M NH4Ac
Bring volume to 400 ml with ddH2O.

DNA Isolation

Perform in a fume hood with protective goggles.

1. Add 300-400 mg dry tissue to a 15mL Falcon Tube (screw cap).
2. Add 7 mL CTAB stock solution (with freshly added β-mercaptoethanol). Vortex to mix well.
3. Incubate 90 min. at 65°C. Invert every 15 minutes.
4. Remove tubes from the bath and cool 10 min.
5. Add 8mL 24:1 Chloroform: IsoAmyl alcohol.
6. Invert for 10 min. on the rocker.
7. Centrifuge for 10 min. at 3,700 rpm.
8. Pour off the supernatant into a new snap-top tube.
9. Add 5uL of 20 mg/mL Rnase A. Incubate 30 min. at 37°C.
10. Add 4 ml C/I and shake 10 min.
11. Centrifuge for 10 min. at 3,700 rpm.
12. Remove the supernatant with transfer pipette into a new snap-top tube.
13. Add 5 ml isopropanol to precipitate DNA.
14. Remove DNA with glass hook or spin down to pellet the precipitated DNA
15. Re-suspend DNA in 300-500ul nuclease-free water.
16. Transfer to a 1.5mL tube.
17. Quantify DNA using a nanodrop.

DNA Cleanup

1. Add equal volume of phenol:chloroform to DNA sample and mix well/vortex gently.
2. Spin at 8,000rpm for 3 minutes.
3. Transfer the aqueous layer to a new 1.5mL tube and add equal volume chloroform.
4. Spin at 8,000rpm for 3 minutes.
5. Transfer the aqueous layer to a new 1.5mL tube.
6. Add 0.1 volume cold 3M Na-Acetate (pH 5.5) and 2 volumes cold 100% ethanol. Incubate for 30 minutes to 1 hour at -20°C.
7. Spin at 10,000rpm for 10 minutes, 4°C.
8. Remove supernatant and wash with 400uL cold 70% ethanol.
9. Spin at 10,000rpm for 5 minutes and remove ethanol.
10. Air dry for 5-10 minutes.
11. Resuspend DNA in 200uL nuclease-free water.
12. Quantify DNA using a nanodrop.

APPENDIX A.3. Modified DNA extraction using seed chips with sbeadex (LGC Genomics)

Materials necessary for seed chipping:

- Sbeadex maxi plant kit 41602
- 2 Riplate 96-well 1.2 mL polypropylene plates
- Qiagen tungsten carbide beads (P# 69997)
- 24 Qiagen 8-well cap strips (Collection Microtube caps, P# 19566)
- beta-mercaptoethanol (BME)
- RNase A
- Sterilized pure water
- 96-well 500 uL polypropylene plate(s) (FisherBrand)
- 10 racks of P200 tips. Also, a box of Matrix pipettor tips.
- Microplate film, sterile (USA Scientific)

Prep:

1. Place chipped seed and 1 tungsten carbide bead into each well of a 96-well Riplate. Place seed kernel into same well in another 96-well plate. Be sure to keep a well clear for a blank.
2. Preheat incubator at 65°C
3. Add 1% BME to both Lysis buffer PN (add 270 uL) and Binding buffer PN (add 550 uL)
4. Add 1 unit of RNase A to Lysis buffer PN (add 38.4 uL)
5. Grind material to a flour using Mixer Mill for 4 X 45 seconds at 30 Hz. Tear down, reset caps, set back up and repeat until tissue is ground well 4 total cycles or more.

Manual protocol:

1. Add 125 µL **Lysis buffer PN** (with BME and RNase). Set new caps and shake by hand to get powder on sides of plate.
2. Incubate at 65°C for at least 10 minutes then centrifuge at 2 500 g for 10 minutes.
3. Add 260 µL of **Binding buffer PN** (with BME) to a fresh Riplate.
4. Resuspend **sbeadex particles** and add 30 µL to each well containing binding buffer.
5. Transfer 100 uL of lysate to fresh plate containing binding buffer and sbeadex particles. Mix thoroughly (5 times).
6. Incubate for 4 minutes at room temperature.
7. Spin plate for 3-5 min to allow for sbeadex particles to form a pellet.
8. Remove supernatant and discard.
9. Add 200 µL of **Wash buffer PN 1** and resuspend pellet (mix 5 times).
10. Incubate at RT for 10 minutes agitating the sample during the time period. Spin plate for 3-5 min to allow for sbeadex particles to form a pellet. Remove supernatant and discard.

11. Add 200 µL of **Wash buffer PN 2** and resuspend pellet (mix 5 times).
12. Incubate at RT for 10 minutes agitating the sample during the time period. Spin plate for 3-5 min to allow for sbeadex particles to form a pellet. Remove supernatant and discard.
13. Add 200 µL of **pure water** and resuspend pellet (mix 5 times).
14. Incubate at RT for 10 minutes agitating the sample during the time period. Spin plate for 3-5 min to allow for sbeadex particles to form a pellet. Remove supernatant and discard.
15. Add 50 µL of **Elution buffer PN** and resuspend the pellet (mix 5 times).
16. Incubate at 55 °C for 10 minutes, agitating sample during time period.
17. Wait 3 min RT, spin plate for 3-5 min seconds to allow for sbeadex particles to form a pellet.
18. Remove eluate and place into new 96 well plate. Transfer only 40 µL of eluate.

To Clean Beads: rinse immediately with isopropanol, rinse with water, do an acid rinse with HCl, rinse well with water, rinse with 95% EtOH and allow to air dry.

APPENDIX B. Tissue culture plating media

N6 Resting Media (IM)	**1L**	**2L**
N6 Salts (g)	3.98	7.96
L-proline (g)	2.875	5.75
Sucrose (g)	30	60
1 mg/mL 2, 4-D stock (mL)	2	4
ddH_20	800	1600
pH 5.8 with 1M KOH		
Gelzan (g)	3.5	7
Bring to volume (L)	1	2
Autoclave for 20 min		
After cooling, add filter sterilized:		
1000x N6 vitamin stock (mL)	1	2
8.5 mg/mL $AgNO_3$ stock (mL)	0.1	0.2

R1-Regeneration (RM1)	**1L**	**2L**
MS Salts (g)	4.3	8.6
Sucrose (g)	60	120
Myo-inositol (g)	0.1	0.2
ddH_20	800	1600
pH 5.8 with 1M KOH		
Gelzan (g)	3.5	7
Bring to volume (L)	1	2
Autoclave for 20 min		
After cooling, add filter sterilized:		
1000x MS vitamin stock (mL)	1	2
1 mg/mL IAA (mL)	1	2
1 mg/mL Zeatin (mL)	0.5	1
1 mg/mL ABA (uL)	23.22	46.44

R2-Regeneration (RM2)	**1L**	**2L**
MS Salts (g)	4.3	8.6
Sucrose (g)	40	80
Myo-inositol (g)	0.1	0.2
ddH_20	800	1600
pH 5.8 with 1M KOH		
Gelzan (g)	3.5	7
Bring to volume (L)	1	2
Autoclave for 20 min		
After cooling, add filter sterilized:		
1000x MS vitamin stock (mL)	1	2
1 mg/mL IAA (mL)	1	2
1 mg/mL Zeatin (mL)	0.5	1
1 mg/mL ABA (uL)	23.22	46.44

1000x N6 vitamin stock (500 mL)
Dissolve in 500 mL ddH2O
0.5 g thiamine HCL
0.25 g pyridoxine HCL
0.25 g nicotinic acid
1.0 g glycine
Filter sterilize and store in -20 C in 40 mL aliquots, thaw and use over a period of weeks

1mg/mL 2,4-D stock (125 mL)
Weigh 0.123 g 2,4-D
Dissolve in 4 mL 1M KOH
Heat gently. Add up to 125 mL total volume with ddH2O. Store in 4 C.

8.5 mg/mL $AgNO_3$ stock (50 mL)
Weigh out 0.425 g Silver Nitrate. Bring up to 50 mL volume with ddH2O.
Filter sterilize. Cover with foil (light sensitive). Store in freezer.

MS vitamin stock (modified) by Frame et al. (500 mL)
1.0 g glycine
0.25g thiamine HCL

0.25 g pyridoxine HCL
0.025 g nicotinic acid
Dissolve in 500 mL ddH2O
Stock is 1000x
Filter sterilize
Store in -20 C in 40 mL aliquots
Allowed to thaw and use over a period of weeks

1 mg/mL IAA (200 mL)
In 250 mL beaker, dissolve 0.2 g IAA in 50 mL of 95% ETOH
Dilute to 200 mL. Filter sterilize. Cover with foil (light sensitive)

1 mg/mL Zeatin (10 mL)
Dissolve 10 mg and bring up to volume to 10 mL with 1 N NaOH.
Filter sterizlie. Store in -20 C. or buy PhytoTechnologies Product No Z860. Zeatin solution.

1 mg/mL ABA (10 mL)
Dissolve 10 mg and bring up to volume to 10 mL with 1 N NaOH.
Filter sterizlie. Store in -20 C.

Gelzan is from Caisson Labs. CAS# 71010-52-1
MS Salts is from PhytoTechnolgies Lab Product No. M524
or MS salts from Caisson labs: REF: MSP01

MS salts have macro and micro nutrients but no vitamins

APPENDIX C. SAS code

APPENDIX C.1. Chapter 3

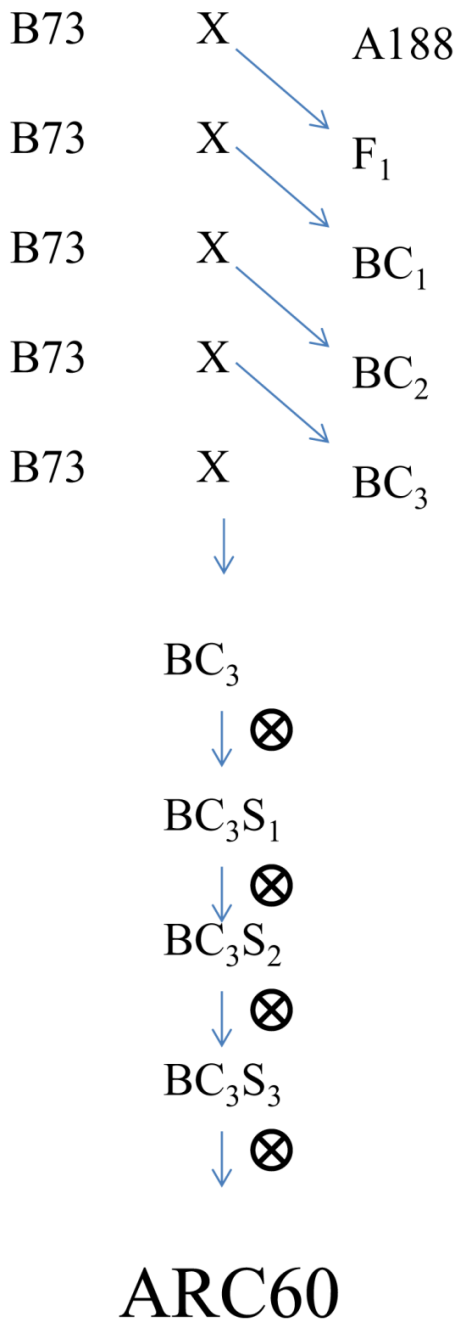
```
proc univariate normal data=expI;
var sum;

 histogram / midpoints=0 to 1 by .1
             lognormal
             weibull
             gamma
             vaxis   = axis1
             name    = 'MyHist';
   inset n mean(5.3) std='Std Dev'(5.3) skewness(5.3)
         / pos = ne  header = 'Summary Statistics';
   axis1 label=(a=90 r=0);

run;

proc npar1way data=expI wilcoxon;
class NIL;
var sum;
run;

proc ttest;
class type;
var sum;
run;
proc univariate data = NIL3;
var total;
   histogram total/normal;
run;

proc glm;
class time gh rep genotype;
model total = time gh rep(gh) genotype time*gh genotype*time genotype*gh genotype*time*gh;
random rep;
lsmeans genotype/pdiff adjust = tukey;
means genotype/lsd;
run;

proc glm;
class time rep genotype;
model total = time rep(time) genotype genotype*time;
random rep(time);
lsmeans genotype/stderr;
run;
```

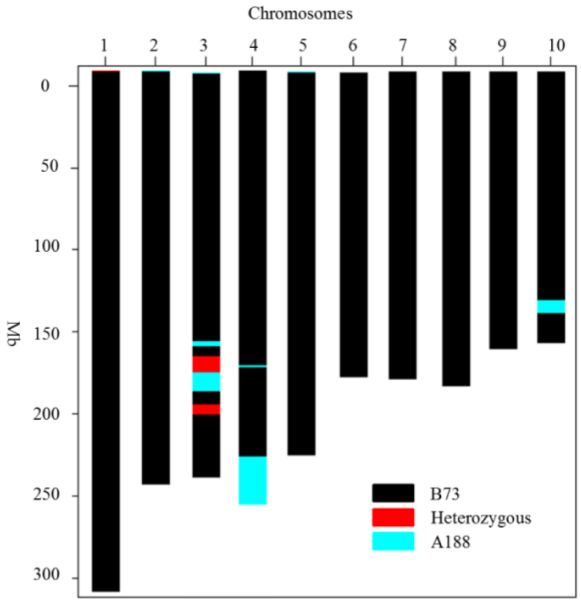
```
proc npar1way data=NIL3_gh22 wilcoxon;
class genotype;
var total;
run;
```

APPENDIX C.2. SAS code for Chapter 4 study on fine-mapping

```
proc glm;
class PZE_103105125;
model PRL = PZE_103105125;
means PZE_103105125;
run;

proc print; run;
proc npar1way data=Ch4_all wilcoxon;
class geno;
var PRL;
run;

proc glm;
class time gh rep geno;
model PRL = time gh rep(gh) geno time*gh geno*time geno*gh geno*time*gh;
random rep(gh);
lsmeans geno/pdiff adjust = tukey;
means geno/lsd;
run;

proc univariate normal plot data=Ch4_all;
var PRL;
   histogram PRL/normal;
   Title 'PRL distribution';
 run;

proc corr;
run;
proc print; run;


/*Table 4.6 test for normality*/
proc univariate normal plot data=Ch4_all;
var E_SE;
   histogram E_SE/normal;
   Title 'E_diam distribution';
 run;

 /*ANOVA, Table 4.7*/
```

```
input plot gh time env rep geno$  E_diam E_SE L_diam L_SE
EH PH ears tiller DH DPlate DTr Days1Rest Days2Rest DaysR1 source_row$;
proc glm;
class time gh rep geno;
model L_diam = time gh rep(gh) geno time*gh geno*time geno*gh geno*time*gh;
random rep(gh);
lsmeans geno/pdiff adjust = tukey;
means geno/lsd;
run;

/*To get Wilcoxon rank sums, Table 4.8*/
proc npar1way data=Ch4_all wilcoxon;
class geno;
var E_SE;
run;
```





APPENDIX D. ARC60 line development of BC_3S_3 population in a backcross-derived population between maize lines B73 and A188 and karyotype using 55,000 single nucleotide polymorphic markers

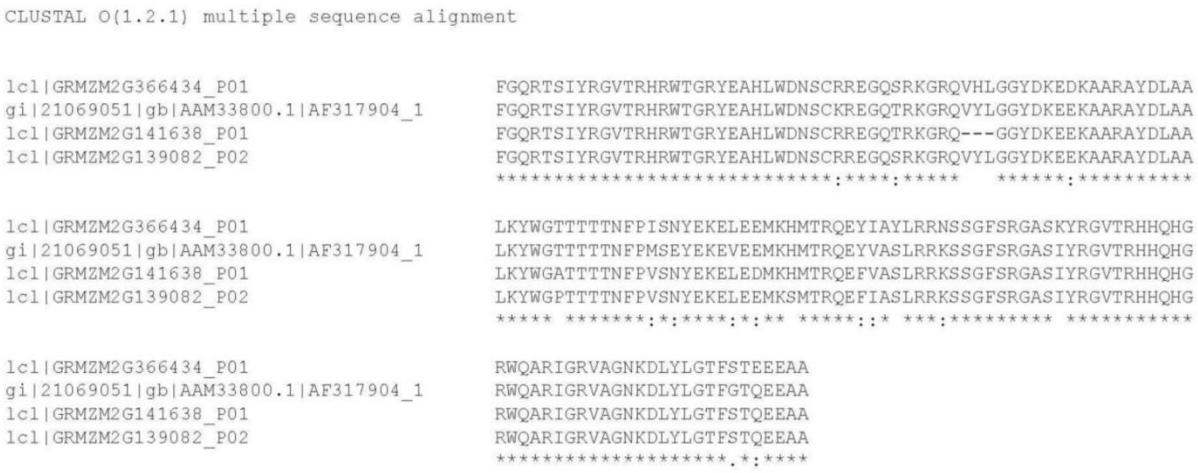
APPENDIX E. Chapter 2 Supplemental Material

Supplemental Table 1. Summary data on transcripts detected. A summary of the number of reads mapped for each sample of immature zygotic embryos from two ears of maize inbred line A188 collected at 0, 24, 36, 48, and 72 h after placement on tissue culture initiation medium.

Ear	Time point	Read Count	Unique Mapping	Duplicate Mapping	Unmapped	Percent Unique Mapping	Percent Duplicate Mapping	Percent Mapped	Percent Unmapped
Ear 1	0h	13,658,058	9,369,648	754,081	3,534,329	68.60%	8.05%	74.12%	25.88%
Ear 2	0h	12,694,970	8,337,899	670,398	3,686,673	65.68%	8.04%	70.96%	29.04%
Ear 1	24h	15,745,857	9,848,683	806,567	5,090,607	62.55%	8.19%	67.67%	32.33%
Ear 2	24h	10,955,885	7,058,441	579,969	3,317,475	64.43%	8.22%	69.72%	30.28%
Ear 1	36h	16,445,774	10,478,756	861,399	5,105,619	63.72%	8.22%	68.95%	31.05%
Ear 2	36h	23,712,334	16,197,689	1,335,271	6,179,374	68.31%	8.24%	73.94%	26.06%
Ear 1	48h	36,048,894	24,978,252	2,020,854	9,049,788	69.29%	8.09%	74.90%	25.10%
Ear 2	48h	17,467,759	12,119,931	983,846	4,363,982	69.38%	8.12%	75.02%	24.98%
Ear 1	72h	13,827,491	9,671,879	792,472	3,363,140	69.95%	8.19%	75.68%	24.32%

Supplemental Table 2. Correlation of transcript abundance between biological replicates across time. Pearson's correlation of transcript abundance estimates measured as fragments per kilobase of exon model per million fragments mapped (FPKM) between samples of immature zygotic embryo explants from two maize ears of inbred line A188 collected at 0, 24, 36, 48, and 72 h after placement on tissue culture initiation medium.

	Ear 1 0h	Ear 2 0h	Ear 1 24h	Ear 2 24h	Ear 1 36h	Ear 2 36h	Ear 1 48h	Ear 2 48h	Ear 1 72h
Ear 1 0h	1	0.9748	0.2587	0.2638	0.5019	0.5806	0.6358	0.6411	0.6835
Ear 2 0h	0.9748	1	0.2570	0.2625	0.4889	0.5667	0.6176	0.6332	0.6640
Ear 1 24h	0.2587	0.2570	1	0.9926	0.7733	0.7494	0.5931	0.5856	0.4867
Ear 2 24h	0.2638	0.2625	0.9926	1	0.7801	0.7564	0.5987	0.5920	0.4904
Ear 1 36h	0.5019	0.4889	0.7733	0.7801	1	0.9643	0.9096	0.8694	0.7974
Ear 2 36h	0.5806	0.5667	0.7494	0.7564	0.9643	1	0.9369	0.9255	0.8569
Ear 1 48h	0.6358	0.6176	0.5931	0.5987	0.9096	0.9369	1	0.9708	0.9587
Ear 2 48h	0.6411	0.6332	0.5856	0.5920	0.8694	0.9255	0.9708	1	0.9566
Ear 1 72h	0.6835	0.6640	0.4867	0.4904	0.7974	0.8569	0.9587	0.9566	1



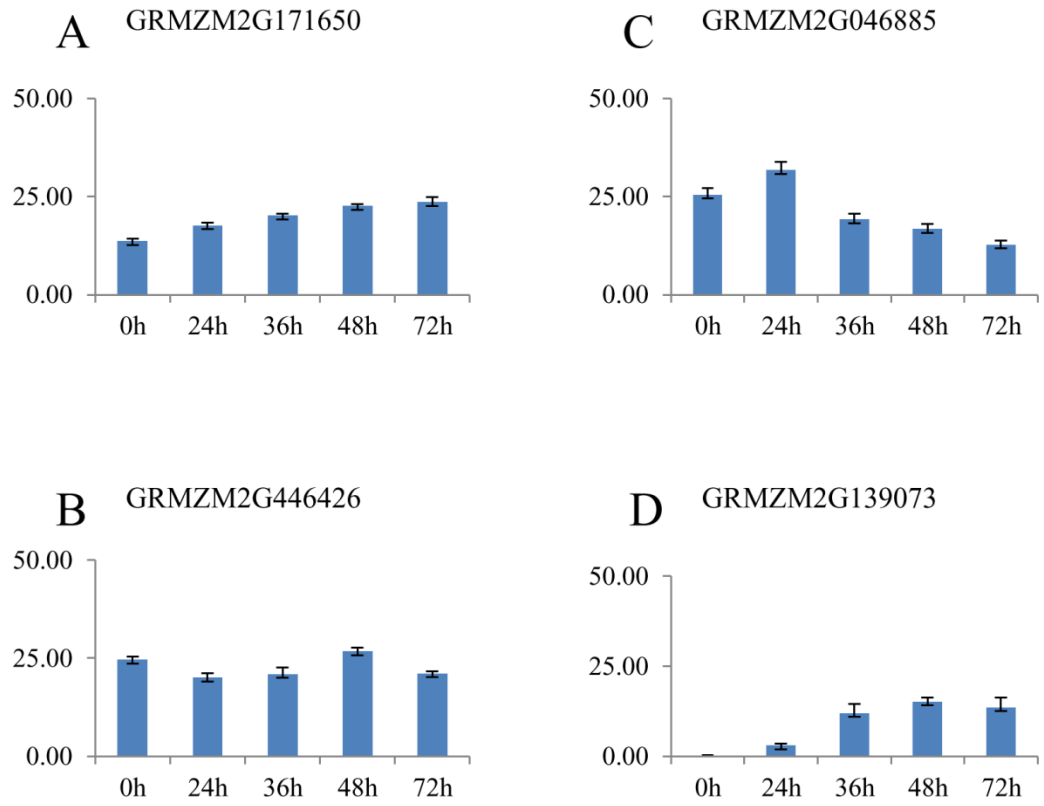
Supplemental Figure 1. Sequence alignment to determine sequence similarity of BABY BOOM in maize. Multiple sequence alignment of the conserved 147 amino acid sequence of BABY BOOM1 (gene accession AF317904) and three maize annotated proteins with high sequence similarity: GRMZM2G366434_P01, GRMZM2G141638_P01, and GRMZM2G139082_P02

```
CLUSTAL O(1.2.1) multiple sequence alignment

gi|15987518|gb|AAL12005.1|AF400124_1      -------------------------------------------MDNFLPFPSSNANS--V
lcl|GRMZM2G405699_P01                     MGQMGGPDGDGPHHQYHYQALLAAVQNPSQGLHVPLHAGAGAPAAGPGPRPGADADASST
                                                                                      * *.::*::  .

gi|15987518|gb|AAL12005.1|AF400124_1      QELSMDPN----NNRSHFTTVPTYDHHQAQPHHFLPPFSYPVEQMAAVMN-----PQPVY
lcl|GRMZM2G405699_P01                     HNANATPHSQPPRAFTDWSASNSAFAAQPAPATTNTPFHYNLSQSYALWTHYMLNKNVSY
                                          :: .  *.    .  :.:::  :    *  *     ** * :.*  *: .      :  *

gi|15987518|gb|AAL12005.1|AF400124_1      LSECYPQI-------PVTQTGSEFGSLVGNPCLWQERGGFLDPRMTKMARI---------
lcl|GRMZM2G405699_P01                     STYSTPHEPLRHTHIPDKYSGCAFSLGFDSF-TTMSLGPNICANMTPMERSISAKEPENS
                                           : . *:       * . :*. *.  . .     . *  :  .** * *

gi|15987518|gb|AAL12005.1|AF400124_1      ------NRKNAMMRSRNNSSPNSSPSELVDSKRQLMMLNLKNNVQISDKKDSYQQSTFDN
lcl|GRMZM2G405699_P01                     EDLPTVVRSSDEMDTRNSGD----------VRRD-TVDTLP-ESKQSHESCASVSNKFDS
                                                *..  * :**...           :*:  : .*  : : *.:. :  ...**.

gi|15987518|gb|AAL12005.1|AF400124_1      KKLRVLCEKELKNSDVGSLGRIVLPKRDAEANLPKLSDKEGIVVQMRDVFSMQSWSFKYK
lcl|GRMZM2G405699_P01                     GEYQVILRKELTKSDVANSGRIVLPKKDAEAGLPPLVQGDPLILQMDDMVLPIIWKFKYR
                                           : :*: .***.:***.. *******:**** ** * : : :::** *:.    *.***:

gi|15987518|gb|AAL12005.1|AF400124_1      FWSNNKSRMYVLENTGEFVKQNGAEIGDFLTIYEDESKNLYFAMNGNSGKQNEGRENESR
lcl|GRMZM2G405699_P01                     FWPNNKSRMYILEAAGEFVKTHGLQAGDT-------------------------------
                                          ** *******:** :***** .* : **

gi|15987518|gb|AAL12005.1|AF400124_1      ERNHYEEAMLDYIPRDEEEASIAMLIGNLNDHYPIPNDLMDLTTDLQHHQATSSMTPEDH
lcl|GRMZM2G405699_P01                     ------------------------------------------------------------


gi|15987518|gb|AAL12005.1|AF400124_1      AYVGSSDDQVSFNDFEWW
lcl|GRMZM2G405699_P01                     ------------------
```

Supplemental Figure 2. Sequence alignment to determine sequence similarity of LEAFY COTYELODN2 in maize. Sequence alignment between LEAFY COTYELODN2 in Arabidopsis (gene accession AF400124) and maize protein GRMZM2G405699_P01.

```
CLUSTAL O(1.2.1) multiple sequence alignment


gi|2160756|gb|AAB58929.1|      IGKGGAGIVYRGSMPNNVDVAIKRLVGRGTG-RSDHGFTAEIQTLGRIRHRHIVRLLGYV
lcl|GRMZM2G141517_P01          IGKGGAGIVYKGTMPDGEHVAVKRLSSMSRGSSHDHGFSAEIQTLGRIRHRYIVRLLGFC
                               **********:*:**:  .**:*** . . *   ****:*************:******:

gi|2160756|gb|AAB58929.1|      ANKDTNLLLYEYMPNGSLGELLHGSKGGHLQWETRHRVAVEAAKGLCYLHHDCSPLILHR
lcl|GRMZM2G141517_P01          SNNETNLLVYEFMPNGSLGELLHGKKGGHLHWDTRYKIAVEAAKGLSYLHHDCSPPILHR
                               :*::****:**:************.*****:*:**:::********.******** ****

gi|2160756|gb|AAB58929.1|      DVKSNNILLDSDFEAHVADFGLAKFLVDGAASECMSSIAGSYGYIAPEYAYTLKVDEKSD
lcl|GRMZM2G141517_P01          DVKSNNILLDSDFEAHVADFGLAKFLQDSGASQCMSAIAGSYGYIAPEYAYTLKVDEKSD
                               ************************* *..**:***:**********************

gi|2160756|gb|AAB58929.1|      VYSFGVVLLEL
lcl|GRMZM2G141517_P01          VYSFGVVLLEL
                               ***********
```

Supplemental Figure 3. Sequence alignment to determine sequence similarity of CLAVATA in maize. Sequence alignment of the 191 amino acid translated sequence representing the catalytic domain of protein kinases superfamily of CLAVATA (CLV1) in Arabidopsis (gene accession U96879) and the maize protein GRMZM2G141517_P01.



Supplemental Figure 4. Gene expression trends in early somatic embryogenesis of maize genes that are similar to AGL15. Fragments per kilobase of exon model per million fragments mapped (FPKM) of maize genes with high sequence similarity to Arabidopsis gene AGL15 (gene accession U22528) associated with embryogenic callus induction expressed in immature zygotic embryos of maize inbred line A188 at 0, 24, 36, 48, and 72h after placement on culture initiation medium. Genes shown include (A) ZmMADS69 (GRMZM2G171650), (B) ZmMADS52 (GRMZM2G446426), (C) ZmMADS73 (GRMZM2G046885), and (D) SILKY1 (GRMZM2G139073). (n=4 for 0, 24, 36, and 48 h include technical and biological replicates; n=2 for 72 h include only technical replicates)