Genetic Fine-Mapping of a Quantitative Trait Locus (QTL) Associated with Embryogenic Tissue Culture Response and Plant Regeneration Ability in Maize (Zea mays L.)

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ABSTRACT
Embryogenic and regenerable tissue cultures are widely utilized in plant transformation, clonal propagation, and biological research applications. Germplasm utilized in those applications are limited, however, due to genotype-dependent culture response. The goal of this study was to identify genomic regions controlling embryogenic and regenerable tissue culture response in the globally important crop, maize (Zea mays L.), toward the long-term objective of developing approaches for genotype-independent plant genetic engineering and clonal propagation systems. An inbred maize line, WCIC2, nearly-isogenic to reference inbred B73, was developed by phenotypic selection and molecular marker analysis. WCIC2 has over 50x increase in tissue culture response relative to the recurrent parent, B73. This line was used to genetically fine-map a region on chromosome 3 controlling embryogenic and regenerable tissue culture response to a 23.9 Mb region. WCIC2 and derivatives will be useful materials to enable maize research in a genetic background similar to B73, and our genetic mapping results will advance research to identify causal genes controlling somatic embryo formation and plant regeneration in maize.

Core Ideas
• Many plant transformation/propagation applications rely on embryogenic cultures.
• Gene regions controlling embryogenic response were identified via fine mapping.
• A culturable line near isogenic to maize inbred B73 was developed.
• Results enable discovery and manipulation of tissue culture response genes.

Tissue cultures capable of forming somatic embryos from which plants can be regenerated (embryogenic and regenerable cultures) are widely utilized in crop biological and genomic research, clonal propagation systems, and in transformation and gene editing-based crop improvement approaches. Understanding the genetic basis of tissue culture response is essential for developing strategies to enhance plant propagation efficiency, while minimizing genetic constraints. The results presented here contribute to the genetic dissection of somatic embryogenesis and plant regeneration in maize, elucidating the genomic regions controlling this vital trait.

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Abbreviations: AGL15, Agamous-Like-15; BBM, Babyboom; DAT, days after tissue culture initiation; DH, doubled haploid inbred line; MS, Murashige and Skoog; QTL, quantitative trait locus; RM1, Regeneration Medium 1; RM2, Regeneration Medium 2; RPL, number of regenerated plantlets; SERK, Somatic Embryogenesis Receptor-Like Kinases; SE, somatic embryogenesis; SE/ZE, number of zygotic embryos displaying somatic embryogenesis; WUS, Wuschel; ZE, zygotic embryos.
improvement applications. Friable embryogenic cultures (Type II) have been the preferred type of regenerable tissue cultures for utilization in many crop transformation protocols, in studies investigating plant embryo development, and in high throughput clonal propagation and synthetic seed applications (Loyola-Vargas and Ochoa-Alejo, 2016; Tripathi, 2017). In most species, however, only a minority of the genotypes tested have been found capable of routinely and efficiently developing these types of embryogenic and regenerable cultures under standard culture conditions. This genotype-dependent culture response can significantly hinder crop functional genomics research, propagation efforts, and biotechnological approaches to crop improvement (Altpeter et al., 2016).

Deciphering the genes and gene networks controlling differential embryogenic and regenerable response in tissue culture would enhance basic understanding of the developmental processes and pathways involved in somatic embryo formation, and aid in the development of improved clonal propagation and crop transformation systems. Investigations spanning an array of plant species such as *Arabidopsis*, maize, cotton, soybean, wheat, pine, and coffee have used reverse genetics approaches to identify several genes having variable effects on somatic embryo formation (Che et al., 2006; Elhiti et al., 2013; Pandey and Chaudhary, 2015; Trontin et al., 2016). The types of genes thus far characterized as contributing to specific aspects of somatic embryo development include, for example, genes encoding transcription factors (e.g., Babyboom, BBM; Wuschel, WUS; Somatic Embryogenesis Receptor-Like Kinases, SERK; Leafy Cotyledon; Agamous-Like-15, AGL15), stress response proteins (e.g., Germin-Like Proteins; Glutamine-S-Transferases) and hormone transport and regulatory proteins (e.g., Pinformed1, PIN1) (reviewed in Cetz-Chel and Loyola-Vargas, 2016; and in Nic-Can and Loyola-Vargas, 2016). Overexpression of genes such as AGL15, BBM, SERK, and WUS, either alone or in combination, has resulted in differential levels of enhanced somatic embryogenesis with, in some cases, reduced genotype-dependent culture responses (Arroyo-Herrera et al., 2008; Bouchabké-Coussa et al., 2013; Boultier et al., 2002; Florez et al., 2015; Hecht et al., 2001; Heidmann et al., 2011; Lowe et al., 2002; Srinivasan et al., 2007; Zheng et al., 2013). Recent enhancement of embryogenesis and transformation efficiency across maize genotypes and target tissues via tailored expression of BBM and WUS2 (Lowe et al., 2016) demonstrated the successful application of basic knowledge of the genetic mechanisms underlying somatic embryogenesis to overcome practical hurdles in crop tissue culture and transformation.

While the above studies have demonstrated enhanced somatic embryogenesis and transformation efficiency via ectopic expression of those specific genes, most reported that results and efficiencies significantly varied among genotypes and/or target tissues, leading to the conclusion that additional, yet unidentified, genetic control factors are likely involved in the processes of somatic embryogenesis and plant regeneration. Identification and characterization of those genes and their interacting networks would not only improve basic understanding of mechanisms controlling the process of somatic embryogenesis, but would also contribute to further development and refinement of strategies to enhance the efficiency of embryogenic and regenerable tissue culture response, and to elicit the response in a genotype-independent manner.

One forward genetics approach toward identification of novel genetic factors affecting embryogenesis and plant regeneration has been through quantitative trait locus (QTL) genetic mapping and analysis using plant populations and lines segregating for tissue culture response. The QTL mapping strategies have been conducted across several plant species to successfully identify genomic regions associated with tissue culture response in crops such as maize, soybean, rice, cotton, and others (Armstrong et al., 1991; Flores Berrios et al., 2000; Bolibok and Rakoczy-Trojanowska, 2006; Bolibok et al., 2007; Hisano et al., 2017; Lowe et al., 2006; Nishimura et al., 2005; Song et al., 2010; Taguchi-Shiobara et al., 2006; Ting et al., 2013; Xu et al., 2015). In some cases, marker-assisted breeding was utilized to transfer the high culture response QTL to nonresponsive genotypes, imparting improved tissue culture response into the germplasm (Lowe et al., 2006). Another study reported map-based identification and cloning of a gene encoding ferredoxin-nitrite reductase that was contained within the QTL and subsequently shown to have a significant effect on regeneration response in rice (Nishimura et al., 2005). Still, there are gaps in knowledge on the genetic factors affecting embryogenesis and plant regeneration in maize and other plant species. We initiated research aimed at identification and validation of novel genes underlying embryogenic and regenerable tissue culture response in the globally important crop, maize, via a QTL fine-mapping approach.

In the public sector, the most highly utilized line for genetic engineering is Hi-II (pronounced “high 2”), with other lines such as B104, A188, and H99 also used to a lesser degree. The highly embryogenic and regenerable maize germplasm, “Hi-II(A×B)”, more generally referred to as “Hi-II”, is generated by a cross between related inbred lines designated “Hi-II Parent A” (or “Hi-II A”) and “Hi-II Parent B” (or “Hi-II B”) (Armstrong et al., 1991). The parent lines were originally produced from a cross between the highly embryogenic and regenerable maize inbred line, A188, to the current maize genome reference inbred, B73. A188 has poor agronomic characteristics, while B73 is a key founder of U.S. dent commercial maize germplasm. A188 is highly responsive to the tissue culture process generating 100% efficiency in observed somatic embryogenic culture response per zygotic immature embryo (SE/ZE) plated in tissue culture (Hodges et al., 1986). B73 has a very low embryogenic and regenerable tissue culture response under standard conditions, with only a 2% efficiency in immature zygotic embryos capable of regenerating green plantlets (Armstrong et al., 1991). The two parental Hi-II lines, “Hi-II A” and “Hi-II B”, derived from the same *F₂* plant from the cross of A188 and B73, were selected for high tissue culture response and improved plant vigor (Armstrong et al., 1991).
Common practice for maize tissue culture is to plant Hi-II (A × B) F₁ seed, produced from the cross of the Hi-II A and Hi-II B lines, and self-pollinate F₁ plants to generate seed containing F₂, immature embryos for utilization in embryogenic culture initiation and genetic engineering applications. Use of Hi-II germplasm in place of the highly embryogenic, regenerable line, A188, is preferable due to the increased vigor of the donor plants and cultures, and the added benefit of possessing a genetic background that is more like the reference inbred line, B73. Although Hi-II is highly efficient in forming embryogenic callus and green plantlets in vitro, there are some disadvantages for use of the germplasm in genetics research and maize crop improvement applications. Hi-II is a hybrid, and when conducting functional genomics testing via transformation, there is genetic background segregation for many quantitative traits among events derived from different F₂ zygotic embryos and within families derived from primary transgenics. For most research and downstream breeding purposes, it is desirable to work within an inbred background. For B73, this could be achieved either by identifying tissue culture processes that would improve response, or by development of a nearly-isogenic B73-type line with introgressions from a line such as A188 that confer tissue culture response.

Investigations aimed at understanding the genetic factors controlling embryogenic and regenerable tissue culture response specifically in maize have utilized genetic mapping approaches to identify regions significantly associated with culture response (Table 1). Meta-analysis of these QTL studies identified several regions common across two or more studies. Results in one study using the A188 × B73 bi-parental mapping population (Armstrong et al., 1992) were similar to another study involving a backcross-derived mapping population between a non-regenerable maize inbred line, FBL, also a stiff stalk line, and the highly regenerable Hi-II (A × B) hybrid (Lowe et al., 2006). In the A188 × B73 mapping study, one DNA marker on chromosome 3, p40 (or agrp40) was used to retain the A188 segment. The p40 marker is estimated to a physical position between 183,798,726 and 184,266,900 based on the nearest loci genetically mapped in the IBM2 population 2008 Neighbors map (Lawrence et al., 2004). Similarly, in another study where regeneration ability (likely originating from A188 through crossing to the Hi-II hybrid) was mapped, markers on maize chromosome bin 3.05 to 3.07 were associated with culturability in BC₁ derived embryos from an FBL × (Hi-II × FBL) backcross. One marker, bnlg1160, was estimated between 187,043,888 and 188,417,593 (Andorf et al., 2010). The QTL reported for tissue culture traits involving other genotypes including H99 and two Chinese maize genotypes, 18 through 599 and Huangzao4, also include loci on chromosome 3 attributing to genetic contributions to culture response (Armstrong et al., 1992; Guangtang et al., 2006; Krakowsky et al., 2006; Lowe et al., 2006; Pan et al., 2006; Zhang et al., 2006).

This study builds on this body of research on QTL identified on chromosome 3 in maize tissue culture that are associated with improved somatic embryogenesis and regeneration capacity in B73. This research was conducted to advance toward the long-term goal of identifying genes affecting embryogenic capacity and regeneration ability in maize. Identification and characterization of genes conferring efficient embryogenic and regeneration response will enhance the understanding of the genetic mechanisms underlying somatic embryogenesis, and will aid in the development of germplasm with enhanced tissue culture response or the design of genotype-independent tissue culture systems.

**MATERIALS AND METHODS**

**Plant Genetic Parent Stocks**

The inbred lines used to initiate this study were A188, which has a high embryogenic and regenerable tissue culture response, and B73, which has low regeneration potential. A188 (Ames22443), was derived from a cross between Silver King and N.W. Dent. B73 (PI 550473), was selected from an Iowa Stiff Stalk Synthetic recurrent selection population. The hybrid, Hi-II (A×B), also generally referred to as “Hi-II”, is the germplasm most often targeted for public maize transformation applications. The Hi-II inbred parent lines (Hi-II A and Hi-II B) and the Hi-II (A×B) hybrid are available from the Maize Genetics Stock Center (http://maizecoop.cropsci.uiuc.edu) (Lawrence et al., 2004) as accessions: Hi-II A (T0940A), Hi-II B (T0940B), Hi-II (A×B) (T0940C).

**Near Isogenic Line (NIL) Development**

Near isogenic line development was conducted as shown in Fig. 1. Briefly, a population of A188 × B73 BC₁S₅ lines (n = 134) were assayed for culture response in replicated experiments, and putative culture response associated QTLs were
identified on chromosomes 1, 3, 4, and 10 using 89 SSR markers (Cook, 2009). Out of the 134 lines screened, one line, designated as WCIC1, displayed phenotypically friable, fast growing callus with high rates of somatic embryogenesis and plant regeneration, comparable to Hi-II. WCIC1 contained A188 alleles in the putative QTL regions as described in previous QTL studies (Table 1, Supplemental Table S1, Supplemental Fig. 1). Additional backcrossing and selfing with WCIC1 to the recurrent parent, B73, combined with replicated phenotypic screening and additional SNP marker analysis revealed significant QTLs associated with embryogenic and regenerable response, with the QTL explaining most of the variation located on chromosome 3 in bin 3.06 (Cook, 2009). A doubled-haploid line much more similar to B73, called WCIC2, was identified from haploid lines derived from (WCIC1×B73)BC5S1 and selected based on its nearly isogenic genotype (Supplemental Fig. 1), high embryogenic tissue culture response and plant regeneration rate as compared to B73 (Fig. 2).

**Genotyping**

A188, B73, WCIC1, Hi-II, Hi-II A, Hi-II B, and WCIC2 were evaluated by Illumina 55k maize SNP chip (Supplemental Table 1, Supplemental Fig. S1). Polymorphic markers were selected for marker-assisted backcrossing with (B73×WCIC1) BC5S. The SNP marker sequences used for KASP genotyping (Semagn et al., 2014) (LGC Genotyping Services, Boston, MA): PZE-103105125 (chr3: 164,821,641), PZE-103107449 (chr3: 166,794,453), PZE-103122471 (chr3: 178,772,856), SYN29001 (chr3: 181,826,658), PZE-103133772 (chr3: 187,789,641), and PZE-103135061 (chr3: 188,705,744) (Supplemental Table S2). Physical positions for SNP loci are based on B73 RefGen v2.

**Selection of Isogenic Recombinant Lines**

Genetic map distances were determined by the number of recombinant genotypes identified in 2243 F2 seed chips in the mapping interval (Fig. 3, Supplemental Table S3). Ninety-nine selected F3 plants homozygous for recombination events in the target region were selected for further study. The F3 plants were self-pollinated and
the F_{3:4} immature embryos were evaluated for embryogenic culture response and plant regeneration potential.

**Tissue Culture Methodology**

Maize immature embryo donor plants were grown at the University of Wisconsin Walnut Street Greenhouse Complex (Madison, WI) under natural light with supplemental lighting conditions (16 h light: 8 h dark). The average light level one meter from the greenhouse floor was 580 µmoles m^{-2}s^{-1} from artificial light provided by 1000-W high pressure sodium bulbs. Greenhouse temperatures were set at 28°C during the day and 22°C at night (±2°C). Maize embryogenic tissue culture initiation using immature zygotic embryo explants was as previously described (Salvo et al., 2014). Subsequent embryo culture maintenance and transfer from initiation to regeneration medium was as follows: At 10 d, the elongating coleoptile was excised and the embryos were transferred onto fresh initiation medium. Callus was transferred onto fresh initiation medium every 2 weeks for a total of 38 d at which time, after the second subculture, only the 10 highest responding embryos (based on callus diameter and visible embryogenic mass) were selected for continued subculture. The selected embryogenic tissue was then transferred to Regeneration Medium 1 (RM1) as previously described (Frame et al., 2006) with the following modifications: RM1 consisted of 4.3 g/L Murashige and Skoog (MS) salts (Caisson Labs, Smithfield, UT) under natural light with supplemental vitamin stock, 1 mL/L 1000X modified 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 3.5g/L Gelzan (Caisson Labs, Smithfield, UT). Filter sterilized hormone stock solutions were then added to the RM1 after autoclaving to reach the final concentrations of 1 mg/L IAA, 0.5 mg/L zeatin, and 0.023 mg/L ABA. The RM1 plates containing the transferred tissues were kept in a plant culture incubator in the dark at 28°C for 10 to 14 d. 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 modified MS vitamin stock, 3.5g/L Gelzan (Caisson Labs, Smithfield, UT) and hormone stock solutions. Plates containing transferred tissues were stored in an incubator at 28°C with 16 h light: 8 h dark photoperiod to allow for shoot formation. Light intensity (at 100–150 µmol m^{-2}s^{-1}) was supplied by 17 W Phillips F17T8 bulbs set at 16 cm above the plates. Tissue was kept on RM2 for 14 d.

**Phenotypic Analysis and Embryogenic Callus Selection**

Tissue culture traits were measured at three time points to track development and growth of embryogenic callus formation (Supplemental Fig. 2, Supplemental Fig. 3). The first time point was to measure culture response and growth during the early weeks of culture initiation. Callus cultures were phenotyped 24 d after tissue culture initiation (24 DAT) which began with the placement of zygotic embryos onto culture initiation/maintenance medium. The second time point was to measure embryogenic response and callus growth rate during the maintenance phase of culture, 38 d after tissue culture initiation (38 DAT). The third time point for phenotypic analysis was 76 DAT to measure number of green plantlets produced after two weeks on RM2 (Supplemental Fig. 2). At 24 DAT and 38 DAT, callus cultures derived from three zygotic embryos (ZE) were chosen at random for callus diameter measurements and the average was recorded. At these time points, the number of ZE producing callus that displayed somatic embryogenesis (SE) were counted and recorded as SE/ZE. At 38 DAT, embryo selections were based on the zygotic embryos displaying the highest embryogenic potential. Twenty out of 50 embryos per source ear were tested for plant regeneration ability. Regeneration ability, measured 76 DAT, was recorded as the total number of regenerated green plantlets (RPL).

**Experimental Design and Data Analysis**

Two hundred sixty-eight individual seeds were selected from the 2243 B73×WCIC2 F2 seeds that were genotyped, and grown in three greenhouse growing environments along with control plants A188, B73, Hi-II, and WCIC2. Ninety-nine F3 plants were analyzed through tissue culture analysis. In the greenhouse, single plants were arranged in a completely randomized design. Data analysis was conducted on genotype as a fixed effect. To estimate phenotypic effect of isogenic lines, plants that shared a genotype, even if the source seed came from a different mother plant, were grouped as isolines as per marker call at each SNP loci. If one locus was in a different phase as compared to its neighboring SNP locus, the assumption was the interval between the loci was heterozygous (Fig. 4). Data analysis was conducted using SAS v9.3 (SAS Inst. Inc., Cary, NC). Pearson’s correlations for tissue culture analysis were calculated (Table 2). The Goodness-of-Fit test for normality was conducted to analyze data distribution. Summary statistics for isoline genotypes were recorded as average phenotypes with standard deviation using raw data (Table 3). The Kruskal–Wallis test statistic was used.
to determine if there were significant differences between isolate genotypes. Wilcoxon scores for each tissue culture trait using isolate genotypes as a variable were used to rank trait means from high to low for each tissue culture phenotype (Table 4). Finally, a Chi-square test for segregation distortion was used to determine significant marker alleles associated with tissue culture phenotypes (Table 5).

RESULTS

Marker-Assisted Breeding Successfully Identified a New Germplasm Source for Embryogenic Culture-Based Research

Inbred backcross line screening of (WCIC1×B73) B73 lines, and the phenotypic evaluation of WCIC2 derived from the WCIC1×B73 backcross populations, led us to proceed with a fine-mapping experiment. To identify WCIC2, we first screened Hi-II, Hi-II A, Hi-II B, A188, B73, and WCIC1 with the Illumina 55k maize SNP chip (Supplemental Table S1). A comparison between A188 and B73 alleles in the Hi-II hybrid, the Hi-II parental lines, and the WCIC1 line led to the development of a doubled haploid inbred line (DH) line for further investigation through phenotypic characterization, backcross breeding, haploid induction and doubling (Supplemental Fig. 1). The DH line, WCIC2, displayed the most suitable genotype for further fine mapping while maintaining its efficiency in embryogenic tissue culture response and plant regeneration ability (Fig. 2). When considering only the 21,811 informative SNP markers that were polymorphic between A188 and B73, the proportion of the genome like B73 in the Hi-II(A×B) hybrid was 69.11%. WCIC1, a progenitor of WCIC2, was 97.54% like B73 and retained 7 regions of A188 introgressions (Supplemental Fig. 1). WCIC2 contained only two small A188-derived regions on chromosome 3 between 164,821,641 and 166,794,453 base pairs, and another between 178,772,856 and 188,705,744 base pairs with 99.22% of the genome being that of B73 (Supplemental Table S1). Concurrent with previous studies (Table 1), there is strong support for the putative QTL regions on chromosome 3 being important for culture response.

Isogenic Lines have Significantly Different Tissue Culture Phenotypes

A total of 99 isolines were evaluated for callus diameter, percent of zygotic embryos displaying somatic embryogenesis (SE/ZE), and number of regenerated plantlets (RPL). A Kruskal–Wallis Test was conducted to determine if isolate phenotypic means were significantly different from each other as determined by tissue culture traits. Using each isolate as a fixed treatment effect and the tissue culture trait as the variable, the Chi-square for all tissue culture traits were highly significant ($P$-value $< 0.001$, df = 14) providing statistical evidence that each isolate performed differently for RPL, SE/ZE, and callus diameter 24 and 38 DAT.

Early Tissue Culture Phenotypes Provide Evidence for Embryogenic Capacity

The target phenotype that describes a successful embryogenic tissue culture response is the ability to form green plantlets from a source explant. The various stages of tissue culture development are distinct, and the tissue culture environment triggers this multi-step process. To describe successful tissue culture response, we took several tissue culture measurements at key stages of the process to determine if the stages of growth into regeneration ability were meaningful in identifying differences between isolines harboring different genes. Pearson’s correlation coefficients found that all the tissue culture traits were significantly correlated with each other ($P < 0.0001$) (Table 2). For RPL, the phenotype with the highest correlation was the percent SE/ZE at 38 DAT ($0.8350$, $P < 0.0001$). One could also consider that since SE/ZE at 38 DAT had the highest correlation with RPL, simple count data could suffice in determining genotypes with efficient tissue culture response in lieu of measuring callus diameter. The traits collected at 24 DAT were also correlated with RPL, however, SE/ZE at 24 DAT displayed the strongest correlation with SE/ZE at 38 DAT ($0.8793$, $P < 0.0001$). Callus diameter traits
24 or 38 DAT were significantly correlated with RPL, although with lower correlation coefficients (0.3780, \( P < 0.0001 \) and 0.7569, \( P < 0.0001 \), respectively). This finding suggests that callus diameter 24 DAT may not provide additional information for a final RPL count and that SE/ZE would be sufficient in describing the progress of embryonic tissue culture growth at 24 DAT. Since tissue culture ability is a multi-stage process data capture needs to be performed throughout the various stages of the process to fully describe possible genes involved. For example, callus diameter and SE/ZE at 38 DAT had the highest correlation (0.9426, \( P < 0.0001 \)) when comparing
Isolines 101, 102, and 107 with an A188 homozygous region between markers SYN29001 and PZE-103122471, in addition to isolate 108, which is heterozygous for this 3053 kb region, all share high mean RPL. This suggests that a common introgression in those lines could be contributing to positive tissue culture response.

**Fine-Mapping Revealed a 3053 kb Region Significantly Associated with Tissue Culture Response**

To further validate differences in isolate genotypes and to further refine the interval that may harbor important genes for tissue culture response, we conducted a Chi-square analysis for segregation distortion that revealed the most significant segment associated with the A188 allele and tissue culture traits as PRL, SE/ZE, and callus diameter, is between markers PZE-103122471 and SYN29001 (Table 5). Both markers show a significant Chi-square \((P < 0.0001)\) with all tissue culture phenotypes and the A188 allele. The PZE-103105125 did not show any significant marker allele associations with tissue culture traits measured in this study. Therefore, PZE-103107449 was only significant for the A188 locus when testing segregation distortion associated with the trait SE/ZE at 24 DAT. The downstream markers PZE-103133772 and PZE-103135061 showed significant association with tissue culture traits as well, ranging from 0.0352 for PZE-103135061 and SE/ZE at 24 DAT to 0.0001 for PZE-103133772 SE/ZE at 38 DAT. These results suggest two possibilities, either that there is more than one gene within this region that is controlling these traits, or the downstream markers are linked to the most significant markers PZE-103122471 and SYN29001 and the significant segregation distortion is a result of linkage rather than an actual effect on the traits. Based on recombination frequencies from the F_2 plants of B73 and WCIC2, the genetic distance between PZE-103122471 and SYN29001 is 4 cM (Fig. 3, Supplemental Table S3). There are perhaps a hundred genes in this region. Previous studies on somatic embryogenesis suggest that multiple genes are involved in the process and that transcription factors are also possible suspects to trigger the type of cellular dedifferentiation that is necessary to regenerate somatic embryos into plantlets (Salvo et al., 2014). Further investigation is needed to determine which gene or genes are required.

**DISCUSSION**

Embryogenic and regenerable plant tissue cultures have been widely utilized for biological and genetic studies, clonal propagation applications, and genetic engineering and crop improvement efforts in plant species across the globe (Loyola-Vargas, 2016). In maize, embryogenic and regenerable culture systems have been a critical component of the majority of past and present public and commercial crop transformation efforts. Those efforts have been hindered, however, by genotype-dependent culture response, triggering considerable research aimed
at understanding the genetic and environmental factors controlling culture response, and attempts to develop genotype independent protocols. Screening for, or generation of, plant genotypes having high embryogenic and regenerable culture response and transformation efficiency has been viewed as an important first step toward wide-spread use of genetic engineering and gene editing technologies that can be realized at a larger scale, not only in maize, but across crop species (Rafael-Prado et al., 2014).

This study showcases the development and validation of a B73-like near isogenic inbred maize line with high embryogenic and regenerable culture response that the global scientific community can utilize for the advancements of studies in basic plant biology and functional genomics research. Since B73 is the maize reference genome of choice, experiments that involve gene editing or plant genome manipulation could be more readily executed and meaningful using the most up to date B73 reference map annotations as a starting point. Since maize line WCIC2 and its progeny are inbred and isogenic to B73, these lines are prime candidates to replace the Hi II(A×B) hybrid for tissue culture based research needs.

Fine-mapping and map-based cloning for a tissue culture candidate gene in rice was validated through genome of choice, experiments that involve gene editing or plant genome manipulation could be more readily executed and meaningful using the most up to date B73 reference map annotations as a starting point. Since maize line WCIC2 and its progeny are inbred and isogenic to B73, these lines are prime candidates to replace the Hi II(A×B) hybrid for tissue culture based research needs.

Table 5. Results of a Chi-square test for segregation distortion for A188 and B73 maize alleles genotyped in a B73 × WCIC2 fine mapping population described by six single nucleotide polymorphic (SNP) markers in a putative QTL region on chromosome 3. Traits associated with embryogenic and regenerable tissue culture response were mean number of regenerated plantlets (RPL), the percent of zygotic embryos displaying somatic embryogenesis 24 and 38 d after tissue culture initiation (DAT) and the average callus diameter 24 and 38 DAT.

<table>
<thead>
<tr>
<th>SNP Marker</th>
<th>Allele</th>
<th>N</th>
<th>RPL</th>
<th>SE/ZE 24 DAT</th>
<th>Callus diameter 24 DAT</th>
<th>SE/ZE 38 DAT</th>
<th>Callus diameter 38 DAT</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mean score</td>
<td>P-value</td>
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<tr>
<td>A188</td>
<td>70</td>
<td>69.49</td>
<td>68.25</td>
<td>0.0053*</td>
<td>70.92</td>
<td>69.89</td>
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<tr>
<td>PZE_103133772</td>
<td>B73</td>
<td>51</td>
<td>49.35</td>
<td>0.0005**</td>
<td>50.02</td>
<td>0.0045*</td>
<td>47.38</td>
</tr>
<tr>
<td>A188</td>
<td>73</td>
<td>67.48</td>
<td>65.95</td>
<td>0.0053*</td>
<td>67.70</td>
<td>67.42</td>
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<tr>
<td>PZE_103135061</td>
<td>B73</td>
<td>48</td>
<td>51.15</td>
<td>0.0053*</td>
<td>52.32</td>
<td>0.0352*</td>
<td>51.05</td>
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</table>
that showed significant allele frequency for the A188 allele. Tissue culture response is a multi-step process from initiation, to maintenance, to regeneration, all of which use different media and/or environments to stimulate tissue and plant growth. Therefore, studying the gene transcripts and expression profiles at later stages during tissue culture development would provide additional useful information toward deciphering the genetic mechanisms underlying embryogenic and regenerable tissue culture response.

CONCLUSIONS

This study sought to increase the resolution in the genetic and physical map of a QTL region associated with embryogenic and regenerable tissue culture response. A near-isogenic, doubled haploid, maize line was developed using marker-assisted selection and single marker trait associations with the long-term goal to identify candidate genes by map-based cloning. Two flanking SNP markers, PZE-103122471 and SYN29001, spanning a 3053 kb interval on chromosome 3, described genotypes that display consistent high tissue culture response in five traits: early callus diameter, early somatic embryogenesis, late callus diameter, late somatic embryogenesis, and plantlet regeneration response. This finding suggests that a major gene, or genes, controlling high embryogenic and regenerable tissue culture response could be identified in this region. Future studies focusing on a candidate gene approach, looking at plausible genes in the 3053 kb region between PZE-103122471 and SYN29001, or mapped subregion, could aid in reaching the ultimate goal of understanding the genetic mechanisms that control somatic embryogenesis and efficient regeneration response in tissue culture in maize and other crops.

Supplementary Material

Supplemental Table S1 contains raw data from SNP molecular marker calls on the Illumina 55K maize SNP chip on inbred lines A188, B73, the Hi-II(A×B) hybrid, Hi-II A, Hi-II B, and WCIC1.

Supplemental Table S2 lists SNP markers on maize chromosome 3 flanking A188-derived genomic segments of a nearly isogenic line, WCIC2.

Supplemental Table S3 provides genetic map information as intervals described by six flanking SNP markers between 164 to 188 Mb on maize chromosome 3 describing the recombination frequency between the markers that were used to Genotype 2243 F2 seeds from a fine-mapping population between maize lines B73 x WCIC2.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

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References


