Quantitative Trait Loci (QTLs) for Intumescence Severity in *Eucalyptus globulus* and Validation of QTL Detection Based on Phenotyping Using Open-Pollinated Families of a Mapping Population

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Abstract

Intumescence is a nonpathogenic physiological disorder characterized by leaf blistering. This disorder can affect growth and development in glasshouses and growth chambers and may be confused with pathogenic diseases. We used quantitative trait loci (QTL) analysis to examine the genetic basis of variation in intumescence severity in *Eucalyptus globulus*, and test for colocation with previously detected QTLs for pathogen susceptibility. QTL analysis used the phenotype means of open-pollinated (OP) families of an outcrossed F₂ mapping family (OP F₃; *n* = 300) of *E. globulus* and the linkage map constructed in the F₂. We validate this phenotyping approach for QTL analysis by assessing

a trait previously used for QTL discovery in the F₂ and showing the same major QTL was detected with the OP F₃. For intumescence severity, five putative QTLs were detected across four linkage groups. Four of these did not colocate with previously reported QTLs for fungal pathogen susceptibility in *Eucalyptus*, suggesting the mechanisms underlying susceptibility to intumescence and to the two fungal pathogens are largely independent. This study demonstrates there is a genetic basis for variation in intumescence severity, reports the first QTL for intumescence severity in plants, and provides a robust framework for investigating the potential mechanisms involved.

Nonpathogenic, blister-like protuberances that develop on leaves (Wolf and Lloyd 1912) are commonly referred to as intumescences or edema. They have been described across a wide range of crop plants, including tomato, sweet potato, geranium, and cassava (Craver et al. 2014b; Morrow and Tibbitts 1987; Williams et al. 2014; Wolf and Lloyd 1912), as well as trees of the genus *Eucalyptus* (La Rue 1933; Pinkard et al. 2006; Warrington 1980). While it remains uncertain whether intumescence formation reflects the same morphological/physiological processes across taxa (Rud 2009; Williams et al. 2014), this disorder generally occurs due to the development of abnormal cells through hypertrophy and/or cell division in the epidermis or mesophyll of a leaf, which may affect growth and lead to tissue necrosis (Craver et al. 2014b; Pinkard et al. 2006; Williams et al. 2014). Intumescences are most commonly reported from plants grown under controlled environments, such as glasshouses and growth chambers, where it appears they result from the interaction of a number of abiotic factors (Craver et al. 2014a). For example, different studies have implicated the interaction of high temperatures and humid conditions often found in glasshouses (Eisa and Dobrenz 1971; Pinkard et al. 2006; Warrington 1980), inadequate UV light quality (Eguchi et al. 2016; Lang and Tibbitts 1983; Williams et al. 2016), and factors such as air quality, plant nutrients, and hormone status (reviewed in Pinkard et al. 2006).

Glasshouses and growth chambers are commonly used to grow plants for research, breeding, and commercial purposes. Intumescences can negatively impact plant growth and survival under these conditions, with potential symptoms including reductions in leaf photosynthesis (Craver et al. 2014b; Pinkard et al. 2006; Roloff et al. 2004), leaf necrosis and senescence (Craver et al. 2014b; Pinkard

et al. 2006), and impacts on leaf water relations (Craver et al. 2014b). Therefore, this disorder can adversely affect experimental results, germplasm screenings, as well as commercial production in glasshouses and growth chambers (Evans et al. 1983). This is particularly important if specific genotypes are more susceptible to intumescence formation, as has been reported in sweet potato (Craver et al. 2014a), potato (Petitte and Ormrod 1986), bat-faced cuphea (Jaworski et al. 1988), and tomato (Sagi and Rylski 1978).

The forest tree *Eucalyptus globulus* Labill. is native to coastal regions of southeastern Australia, but is widely grown for pulpwood in temperate regions of the world, including Australia (Potts and Vaillancourt 2004). In line with its ecological and economic importance, the genetic basis of variation in many traits has been studied (Potts and Vaillancourt 2004). Intumescence formation has been reported in glasshouse-grown seedlings of *E. globulus* (Pinkard et al. 2006); however, to our knowledge, the genetic basis of variation in intumescence formation has never been studied in *E. globulus* or any other forest tree species. The intumescence described by Pinkard et al. (2006) were morphologically quite different to those reported in many other taxa and resembled lenticels (raised pores that allow gas exchange) in their simplest form. They are the result of prolific division and enlargement of de-differentiated parenchyma cells, which protrude into and eventually rupture the epidermis, exposing the internal tissues and facilitating aeration of the leaf tissues. *E. globulus* is susceptible to a number of foliar diseases in many regions where it is grown (Dick and Dobbie 2001; Glen et al. 2007). Screening for susceptibility to foliar pathogens such as myrtle rust (*Austropuccinia psidii*, formerly *Puccinia psidii*) by artificial inoculation requires high relative humidity (Butler et al. 2016), which can also result in the formation of intumescences (Pinkard et al. 2006). Thus, the presence of intumescence may potentially confound studies on the effects of, and susceptibility to, foliar pathogens as well as other types of screenings conducted in controlled environments (Warrington 1980).

Progeny testing is commonly used to phenotype both plants and animals (Kashi et al. 1990; Wenzel and Foroughi-Wehr 1990). In breeding, this method involves testing the genetic worth of an individual by examining the average performance of its progeny. Progeny testing for quantitative trait loci (QTL) analysis was originally advocated as a means of reducing the environmental variance (Lander and Botstein 1989). This technique has been successfully employed to identify QTLs influencing milk production in bulls,

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based on the milking performance of a large number of daughters produced under controlled matings (Georges and Nielsen 1995). However, while controlled mating designs are routinely used for progeny testing in breeding animals and some plant species, progeny testing in forest trees often involves using open-pollinated (OP) offspring and has traditionally been used to estimate heritabilities and breeding values in population-level analyses (Hodge et al. 1996; Johnson and Brudon 1990). Recently, Butler et al. (2016) used phenotyping by progeny testing for QTL analysis in *E. globulus*. In this case, phenotypic information was acquired from OP offspring collected from an F_2 population of trees growing in a field trial. This technique has a number of advantages for long-lived organisms, such as forest trees, that only express traits of interest at certain stages in the life cycle and are not amenable to methods such as clonal propagation that prolong the use of experimental populations for genetic study (Butler et al. 2016). While this technique is conceptually promising, it still requires further experimental validation.

The primary aim of the present study was to investigate the genetic basis of variation in intumescence severity in *E. globulus*. This was done using QTL analysis of an outbred F_2 *E. globulus* family, using phenotypic data from their OP F_3 families. These families were being grown to study QTLs affecting seedling characteristics not assessed in the original F_2 . We also tested whether the outbreak of intumescence had a deleterious effect on seedling growth, and whether QTLs previously reported for pathogen susceptibility could be associated with susceptibility to this disorder. A secondary aim was to test the validity of using OP families from QTL mapping families for phenotyping in QTL analysis, by assessing in the F_3 a trait previously studied in the F_2 (Hudson et al. 2014) and comparing the location, inheritance, and significance of detected QTLs.

Materials and Methods

Plant material and trial design. The QTL analysis was conducted using phenotypic data from the OP F_3 families of a large outcrossed F_2 mapping family of *E. globulus* (Lighthouse F_2 , see Hudson et al. 2014; Fig. 1). The OP F_3 represented a subset ($n =$

300) of the (503) F_2 individuals used in previous QTL and mapping studies (Hudson et al. 2012, 2014), which were reproductively mature when this study was initiated. This F_2 family (614LH/KI440//615LH/TA423) was originally designed to maximize the segregation of ecotypic differences in vegetative phase change. It was constructed from crossing two unrelated F_1 individuals (the parents), each derived from crossing individuals (the grandparents) with precocious (Wilson's Promontory Lighthouse [LH]) and "normal" (from either King Island in Bass Strait [KI] or Taranna in the southeast of Tasmania [TA]) vegetative phase change. This cross design captures the segregation from within and between the provenances of origin. The F_2 family was grown at two sites (Geeveston and Boyer in Tasmania) and OP seed was collected from 115 individuals from the Geeveston site and 185 from the Boyer site ($n = 300$). This seed was used to produce F_3 families that were grown together in a glasshouse during summer-autumn 2016, in a randomized complete block design (Fig. 1). The design comprised 12 randomized blocks, with each F_3 family represented once per block, resulting in each F_2 individual being represented by 12 seedlings in total. The seedlings were grown at a spacing of 100 × 50 mm, in 50 × 50 × 120 mm forestry tubes filled with a potting medium composed of 90% urea-composted pine bark, 5% coarse potting sand, 5% cocoa peat, and slow release (5 to 6 month) low phosphorus Osmocote fertilizer for natives (NPK 16:1.3:9.1), at a rate of 3 kg per cubic meter of potting medium. Seedlings were watered every 1 to 2 days as required. Daily maximum temperature in the glasshouse ranged from 22 to 38°C, and relative humidity ranged from 50 to 90%.

Scoring of intumescence. Leaf intumescence developed following a period of warm weather, resulting in high temperature and high relative humidity in the glasshouse, after approximately 3 months of plant growth when all plants were still in their juvenile phase. Intumescence severity was assessed on each plant, on a scale of 1 to 4: 1 = little to no intumescence or leaf in-rolling; 2 = intumescence and leaf rolling on two leaves or less; 3 = intumescence on most leaves; 4 = all leaves severely affected by intumescence and in-rolled (Fig. 2).

Other trait measurements. After intumescence assessment, steps were taken to mitigate the further development of intumescence in the glasshouse. Ventilation was increased by placing fans inside

Fig. 1. Experimental design showing pedigree of the *Eucalyptus globulus* F_1 parents (LH = Lighthouse, TA = Taranna, KI = King Island) that were crossed to produce the field grown F_2 mapping population ($n = 503$). The F_2 was grown at two sites (Geeveston and Boyer) and these trees were used to generate the linkage map. Seed from 300 open-pollinated F_2 trees were collected and seedlings grown in a randomized complete block design (12 replicate blocks, each family represented once per block) in the glasshouse to produce the F_3 population. Quantitative trait loci (QTL) analysis was based on F_3 family means and the linkage map generated from the F_2 population.

Fig. 2. i) *Eucalyptus globulus* seedlings showing various degrees of leaf intumescence severity, from left to right, least to most severe (1 to 4). Plants with little to no intumescence or leaf in-rolling (1), plants with intumescence and leaf rolling on two leaves or less (2), plants with intumescence on most leaves (3), plants with all leaves severely intumesced and in-rolled (4). ii) Close-up of severe intumescence on the surface of an *E. globulus* juvenile leaf, with in-rolling of the leaf.

the glasshouse and allowing the door and louvers to remain open during the day. Slow release low phosphorus Osmocote for natives (NPK 16:1.3:9.1) was also top-filled into each plant pot at the rate of 1 g per plant to stimulate the production of new growth.

Traits related to growth and morphology were scored following intumescence formation to investigate the effect of intumescence severity on subsequent plant growth (Table 1). Plant height (mm) from the cotyledonary node to the base of the apical bud (height_1), the number of fully expanded leaves (total leaves), and leaf mass per area (g/m²) of the last fully expanded leaf (LMA; Cornelissen and Lavorel 2003) were measured at approximately 6 months of age (3 months after intumescence formation), from two replicates. After an additional 5 months of growth, plant height (height_2) and the number of fully expanded nodes (total nodes) were measured from seven replicates. In addition, the diameter of the stem (mm) at the cotyledonary node (stem diameter) and the thickness (mm) of the last fully expanded pair of leaves (leaf thickness) were measured at this time using electronic calipers. Leaf thickness was measured at the base and midpoint of each leaf in a pair, giving a total of four leaf thickness measurements for each plant. The averages of these four measurements were then calculated and used in subsequent analyses.

The timing of the onset of the ontogenetic transition from juvenile to adult leaves (phase change) was the trait used to validate our approach of phenotyping a QTL mapping population using their OP families. Phase change was scored in the glasshouse at the same time as the later growth measurements (11 months of age) from seven replicates. Due to the relatively young age of the seedlings, this trait was scored as the presence or absence of phase change (1 or 0, respectively). The presence of phase change was determined by the development of petiolate leaves, with subsequent nodes continuing to produce petiolate leaves with an increasing tendency toward an iso-bilateral orientation. The QTL analysis was undertaken using the proportion of individuals in each OP F₃ family that had transitioned from the juvenile leaf type. The QTLs detected using the OP F₃ were compared with those detected using the node of phase change assessed in the larger field-grown F₂ population (reported in Hudson et al. 2014).

Analysis. QTL analysis was conducted in MapQTL 6 (Van Ooijen 2009) using the 391-marker biparental consensus linkage map reported by Hudson et al. (2014) and the F₃ family trait means. This map comprised 341 DArT and 50 microsatellite markers, which were a subset of well-spaced markers selected from a larger 1,060-marker map built in this pedigree to reduce ‘clumping’ of markers without compromising genome coverage (for details of the full and reduced maps, see Hudson et al. 2012, 2014). We used this approach since using the 1,060-marker map would provide very little gain in the power of QTL detection, but would exponentially increase the computational requirements of QTL analysis (Darvasi et al. 1993; Van Ooijen 2009). QTL analyses followed the methods described by Butler et al. (2016). In brief, permutation tests were run in MapQTL 6 to determine logarithm of odds (LOD) significance thresholds (1,000 permutations, Churchill and Doerge 1994). Analyses were undertaken with and without site (Boyer or Geeveston) fitted as a cofactor. Putative QTLs were declared at two different levels, significant

(genome-wide type I error rate <0.05) and ‘suggestive’ (chromosome-wide type I error rate <0.05). QTL analyses were conducted first with interval mapping, using the default parameters of MapQTL 6 (Van Ooijen 2009). For each QTL peak exceeding the suggestive LOD threshold in interval mapping, the closest markers were chosen as cofactors for subsequent restricted multiple-QTL model (rMQM) mapping. rMQM analyses were performed using an iterative approach until: 1) no further QTLs were detected; 2) selected cofactor markers were the closest to each QTL peak; and 3) QTL positions were stable (Van Ooijen 2009). A similar procedure was used for the QTL analysis of the phase change data collected from the full complement (*n* = 503) of field grown F₂. To assess whether the segregation of QTL effects was solely on the maternal or paternal sides, or both, Kruskal-Wallis single marker analyses were conducted in Map QTL 6 (Van Ooijen 2009) and the significance of markers with different segregation adjacent to each significant QTL discovered from rMQM analysis were examined. To test for colocation of our QTLs with those previously detected for foliar fungal pathogens from other eucalypt pedigrees (*Austropuccinia psidii* in *E. grandis* and *Teratosphaeria* spp. in *E. globulus*; Butler et al. 2016; Junghans et al. 2003; see below), the peak location of these previous QTLs was translated to our linkage map using shared markers where possible (Butler et al. 2016), or based on the physical position of the markers in the original publications and the closest marker in our linkage map using the *E. grandis* reference genome (Myburg et al. 2014; Fig. 3). In order to identify positional candidate genes for intumescence severity, the physical location of the marker at the peak of each significant (genome-wide type I error rate <0.05) QTL for intumescence severity was determined on the *E. grandis* genome v2.0 using the JBrowse function on Phytozome v12.1 (<https://phytozome.jgi.doe.gov/pz/portal.html>). Genes within 1 Mb either side of these physical positions are listed (Supplementary Table S1) and plausible candidates, based on the *E. grandis* genome annotation, highlighted. We confined our search for candidate genes to within 1 Mb of the QTL peaks, because the locus (or loci) underlying QTL effects are usually within 2 to 3 cM of the QTL peak, even for QTLs with minor effects (Price 2006); and 1 Mb on the *E. grandis* reference genome corresponds to 3 cM on average in our *E. globulus* linkage maps (Gion et al. 2016).

An analysis of F₃ family differences was conducted using PROC GLIMMIX in SAS (version 9.4), fitting family and replicate as random effects. These analyses were undertaken with and without the site of origin of the F₃ family fitted as a fixed effect. When site was fitted, the random family term was nested within site and used as the error term to test the difference between sites using Walds F statistic. A multinomial model using a cumulative logit link function was fitted for the four point intumescence severity score, a binary model with a logit link function was fitted for the presence/absence of phase change, whereas a Gaussian distribution was used for the other traits (height_1, height_2, total leaves, LMA, total nodes, stem diameter, and leaf thickness). The significance of the random terms was tested using a one-tailed likelihood ratio test. The relationship between traits was further investigated through Spearman’s correlation coefficients, calculated using the *cor* function in R (R Studio

Table 1. Number of replicates measured (no. of reps), grand means, standard deviations (SD), minimum and maximum values for the *Eucalyptus globulus* F₃ family trait means, the one-tailed likelihood of ratio test χ^2 (χ^2), and *P* values for the difference between F₃ families (*n* = 300)

Trait	No. of reps	Grand mean	SD	Min.	Max.	χ^2	<i>P</i>
Intumescence severity (1 to 4)	12	1.98	0.44	1.08	3.08	182.8	<0.00
LMA ^a (g/m ²)	2	67.4	10.67	35	107.9	0.4	0.262
Total leaves	2	13.5	1.6	9	17.6	35.3	<0.001
Height_1 (mm)	2	202	24	93	288	36.9	<0.001
Phase change (0/1)	7	0.15	0.17	0	0.75	9.1	0.001
Height_2 (mm)	7	570	72	353	741	3.7	0.027
Total nodes	7	17.3	1.4	13.2	20.6	55.2	<0.001
Leaf thickness (mm)	7	0.23	0.016	0.18	0.27	2.2	0.068
Stem diameter (mm)	7	5.09	0.32	4.1	5.97	24.5	<0.001

^a Leaf mass per unit area.

Team 2015) based on the F_3 family means. The significance of the correlation values were calculated using the *cor.test* function in R.

Results

Our findings provide two lines of evidence for a genetic component to the variation in intumescence severity in *E. globulus*. First was the statistically significant difference among the F_3 family means (Table 1), which argues that intumescence severity is heritable to some degree and there is genetic segregation occurring within the F_2 mapping population. Second, statistically significant QTLs were detected (Table 2), demonstrating that specific regions of the genome contribute to the variation among these F_3 family means.

Five putative QTLs influencing intumescence severity were detected, located across four linkage groups (Table 2; Fig. 3). Three of these QTLs were significant at the genome-wide level (genome-wide type I error rate <0.05), which individually accounted for 6.5 to 8.9% of the variance in intumescence severity among the F_3 families (Table 2). Two further 'suggestive' QTLs were significant at the chromosome-wide level (chromosome-wide type I error rate <0.05). In combination, the five QTLs explained 31.7% of the variance in intumescence severity in the F_3 families, indicating a substantial genetic basis for variation in intumescence severity. Mixed model analysis of the site effect using F_3 family nested within site as the random

error showed a significant effect of site of origin for intumescence severity (multinomial analysis: $F_{1, 298} = 4.8$, $P = 0.029$) and LMA ($F_{1, 290} = 4.0$, $P = 0.047$), but for no other trait tested. Such a site difference could result from differences in the pollen environment between sites. rMQM analyses were thus conducted both with and without site as a cofactor. However, in the case of intumescence severity, these analyses detected the same QTL, and the difference among the F_3 family means was evident whether the site of F_3 family origin was taken into account (LRT $\chi^2 = 176.7$, $P < 0.001$) or not ($\chi^2 = 182.8$, $P < 0.001$; Table 1). This result indicates that the slight differences between the sites at which the F_2 populations were growing did not have a significant effect on the genetic basis of variation in intumescence severity. No QTLs were detected for LMA, regardless of whether the site was included or not in the model.

We could find no evidence that the severity of intumescence development on the *E. globulus* seedlings adversely affected subsequent growth (Table 3). Significant but weak positive correlations ($r \leq 0.2$, $P < 0.001$) were found among F_3 family means for intumescence severity, the two growth measurements (total leaves and height_1/height_2), as well as LMA, all of which were recorded at 6 months of age (3 months after intumescence formation). Intumescence severity was also weakly positively correlated with the total nodes at 11 months of age ($r = 0.2$, $P < 0.001$); however, it was not correlated

Fig. 3. Location of significant quantitative trait loci (QTLs) detected for intumescence severity (*Qint*) from rMQM analyses in *Eucalyptus globulus* and QTLs previously found for susceptibility to foliar fungal pathogens (*Qmyco* = *Teratosphaeria* spp., *Ppr* = *Austropuccinia psidii*) in this and other pedigrees of *E. globulus* (see Butler et al. 2016) and *E. grandis* (Junghans et al. 2003). QTL bars span 18cM (9cM either side of QTL peak) to show colocating QTLs. For simplicity, only the peak marker positions for previously detected QTLs are shown, the other horizontal lines within linkage groups represent the position of markers in the 1,060-marker linkage map built in this pedigree by Hudson et al. (2014). Only linkage groups on which QTLs were found are shown (9 of 11), numbers above each bar indicate linkage group number. Percentage variation explained (PVE) by each QTL is indicated below the QTL names (excluding *Ppr1*).

with leaf thickness or the other growth traits measured at this time (height and stem diameter), suggesting that intumescence severity had limited long term influence on growth.

Two significant QTLs (genome-wide type I error rate <0.05) were detected for phase change, each located on different linkage groups (Table 2). These QTLs explained 5.7 to 12.5% of the variance in phase change among the F₃ families. Importantly, the most significant QTLs detected in the OP F₃ (LOD 9.46) peaked at the same marker as the major QTLs previously detected in the F₂ generation in the mapping population (Hudson et al. 2014). Phase change was not significantly correlated with intumescence severity (Table 3), and QTLs for these traits did not collocate (Fig. 3).

Browsing the *E. grandis* reference genome within a 1 Mb window on either side of the peak of the three QTLs that were significant at the genome-wide level revealed 207 positional candidate genes. These windows corresponded to 14 to 27% of the estimated 95% confidence interval of each QTL (Churchill and Doerge 1994), based on the relationship between physical distance in the *E. grandis* reference genome and our *E. globulus* linkage maps (see Materials and Methods). Of the 207 positional candidates, 55 are considered plausible candidates based on their putative function in either *E. grandis* or *Arabidopsis thaliana* (<https://phytozome.jgi.doe.gov/pz/portal.html>). These functional candidates included genes involved in abiotic and biotic stress responses such as NBS-LRR genes and receptor-like kinases (RLKs).

Table 2. Quantitative trait loci (QTLs) for intumescence severity (*QInt*) and phase change (*QPC*) detected in *Eucalyptus globulus* using rMQM mapping with site as a cofactor; linkage group (LG), map position (cM), marker closest to the QTL peak (adj. marker), significance (LOD), percentage variance explained (PVE), parent from which the QTL effect segregated (Seg: M = male, F = female, B = both), and physical location in the *E. grandis* reference genome is shown

QTL	LG	cM	Adj. marker ^a	LOD ^b	PVE	Seg.	Physical location (bp) ^c
<i>QInt1</i>	5	72.3	600693	6.98***	8.9	B	52,292,107
<i>QInt2</i>	5	34.4	640749	6.12**	7.8	B	12,007,876
<i>QInt3</i>	3	37.1	641763	5.21**	6.5	B	22,006,080
<i>QInt4</i>	6	121.7	570163	3.6	4.4	M	48,487,073
<i>QInt5</i>	11	91.1	Emb747	2.97	3.6	F	36,974,457
<i>QPC1</i>	3	62.8	639243	9.46***	12.5	B	51,835,180
<i>QPC2</i>	11	36.5	Eg99	4.44***	5.7	M	17,349,501

^a Adjacent marker closest to QTL peak. Numbers denote DArT markers, numbers and letters denote microsatellite (SSR) markers.

^b Peak LOD score for each QTL is shown. Genome-wide significance is indicated by *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$. The remaining QTLs were significant at the suggestive level (chromosome-wide type I error rate < 0.05).

^c Physical location on the *Eucalyptus grandis* genome v2.0, based on the mid-point of the sequence tag of the adjacent marker located by BLAST searches. Where the adjacent marker had no sequence, the closest marker with sequence information was used. The numbering and orientation of linkage groups in this study matches the 11 main pseudomolecules in the *E. grandis* genome assembly.

Discussion

While there are reports of a genetic basis to variation in susceptibility to intumescence in other taxa (Craver et al. 2014a; Eisa and Dobrenz 1971; Petite and Ormrod 1986; Seabrook and Douglass 1998), to our knowledge, this is the first report of QTLs influencing intumescence severity in plants. The large differences in susceptibility to intumescence reported between cultivars of potato and sweet potato (Craver et al. 2014a; Petite and Ormrod 1986) are consistent with the relatively large effect sizes (i.e., >5% variance explained) of the QTLs detected in this study. Nevertheless, the present study suggests that intumescence formation is under multigenic control, with at least several QTLs impacting susceptibility to this disorder.

Water congested leaves not only contribute to intumescence formation (Pinkard et al. 2006), but provide favorable conditions for infection by several bacterial (Graham et al. 1992; Zehr et al. 1996) and fungal plant pathogens (Bald 1952; Johnson 1947), including *Austropuccinia* species (Kozłowski 1978). This highlights the possibility that susceptibility to intumescence may be associated in some way with susceptibility to foliar fungal diseases. To test for such an association, we searched for collocation (defined here as having a peak location within 3 Mb on the *E. grandis* reference genome, which equates to ~9 cM on the *E. globulus* genome on average; Gion et al. 2016) of our QTLs with those previously discovered for susceptibility to two fungal pathogens (Fig. 3); (i) *Austropuccinia psidii* (known as myrtle rust or guava rust), which is a pathogen of global importance that affects a wide-range of host species in the family Myrtaceae, including *Eucalyptus* (Giblin and Carnegie 2014), and (ii) *Teratosphaeria cryptica*, formally included in the genus *Mycosphaerella*, which includes major foliar pathogens of eucalypts worldwide (Carnegie et al. 2011). QTLs for both pathogens were identified in the same pedigree as used in the present study (Butler et al. 2016), as well as in independent *E. globulus* (Freeman et al. 2008) and *E. grandis* (Junghans et al. 2003) pedigrees for *T. cryptica* and *A. psidii*, respectively. The *A. psidii* QTLs were identified by controlled inoculation of shade-house grown F₃ families in Queensland, Australia, while those for *T. cryptica* were identified based on natural infection in field trials in Tasmania (Butler et al. 2016; Freeman et al. 2008).

An association between susceptibility to these fungal pathogens and intumescence severity could occur through shared mechanisms that confer differences in vulnerability to water congestion between individuals, such as variation in cellular structure or growth habit. Alternatively, since both pathogens infect their hosts by directly penetrating the leaf cuticle, rather than entering through the stomata (Park 2000; Xavier et al. 2015), intumescence formation may affect susceptibility to disease through the provision of entry points for infection. In addition, the scoring of susceptibility to foliar fungal pathogens may also include intumescence, due to the superficial resemblance in symptoms (i.e., cell hypertrophy and necrotic spots, see Park 2000). Each of these scenarios could result in the discovery of colocated QTLs for intumescence severity and susceptibility to fungal diseases. However, of the 12 QTLs collectively reported for susceptibility to *A. psidii* and *T. cryptica* in *E. globulus* (Butler et al.

Table 3. Pairwise Spearman's correlation coefficients for phenotypic traits measured in the *Eucalyptus globulus* F₃ ($n = 300$). F₃ family means are used for the phenotypic trait values.^a

	Intumescence severity	Total leaves	LMA ^b	Height_1 ^c	Height_2 ^d	Total nodes	Leaf thickness	Stem diameter
Total leaves	<u>0.13</u>	...	...	...	...	...	...	...
LMA ^b	<u>0.2</u>	<u>0.17</u>	...	...	...	...	...	...
Height_1 ^c	<u>0.19</u>	0.11	0.29	...	...	...	...	...
Height_2 ^d	0.06	<u>0.13</u>	-0.03	0.07	...	...	...	...
Total nodes	0.2	<u>0.20</u>	<u>-0.13</u>	0.05	0.46	...	...	...
Leaf thickness	-0.01	0.05	0.11	0.09	-0.04	-0.11	...	...
Stem diameter	0.02	0.22	0.01	0.03	0.34	0.2	0.33	...
Phase change	0.03	-0.11	0.08	-0.04	-0.06	<u>-0.18</u>	<u>0.15</u>	-0.04

^a Significance values ≤0.05 are italicized and underlined, values ≤0.001 are in bold.

^b Leaf mass per unit area.

^c Height measurement at 6 months of age.

^d Height measurement at 11 months of age.

2016) and *A. psidii* in *E. grandis* (Junghans et al. 2003) only one, a 'suggestive QTL' (chromosome-wide type I error rate <0.05) for *T. cryptica*, collocated with a QTL for intumescence severity on linkage group 3 (Fig. 3). This collocation could be due to one of the above explanations. However, the possibility that discrete loci underlie each QTL cannot be dismissed, particularly as they were discovered in independent pedigrees of *E. globulus*, which reduces the probability that the same loci are polymorphic in each pedigree (c.f. collocated QTLs within a pedigree). In addition, the QTL detected for *T. cryptica* susceptibility was based on natural infection of field trials in (cool temperate) Tasmania, which are unlikely to suffer intumescence, hence it is unlikely that the scoring of disease symptoms was confounded by the presence of intumescence. Clearly, further study is required to determine if the same locus, or discrete loci, underlie the collocated QTLs we highlight; and if they are in fact the same locus, what mechanism underlies this collocation. Nonetheless, the comparison of our QTLs for intumescence with those previously discovered for susceptibility to *A. psidii* and *T. cryptica* in *E. globulus* (Butler et al. 2016) and *A. psidii* in *E. grandis* (Junghans et al. 2003), suggest that they are largely under independent genetic control and that intumescence and disease symptoms have not been confounded when phenotyping in our previous studies of these pathogenic diseases.

Interestingly, despite the general lack of collocation between QTLs for susceptibility to pathogenic diseases and intumescence, the majority of the functional candidate genes detected belonged to the large NBS-LRR family of proteins, which are one of the major classes of disease resistance (R) genes in plants (Christie et al. 2016; DeYoung and Innes 2006; McHale et al. 2006). NBS-LRR proteins play functional roles in the recognition and response to external stimuli, as well as activating the downstream signaling pathways that lead to immune responses and cell death (Christie et al. 2016). In addition, NBS-LRR genes have been found to play a similar role in the detection and response to abiotic stresses such as drought and cold stress, heat shock, and mechanical wounding (Cheong et al. 2002; Chini et al. 2004). The next most abundant candidate genes found were the topless-related proteins (TPLs) and the RLKs, both of which are also involved in the detection and response to abiotic stresses such as heat and osmotic stress (Park et al. 2014; Pauwels and Barbero 2010; Ye et al. 2017). Finally, a small number of genes that are involved in the synthesis of resveratrol were also detected. Resveratrol belongs to a class of compounds that plants produce in response to environmental stress and has been found to accumulate in response to high levels of UV light, physical harm to plant tissues, or pathogen infection (Chung et al. 2003). While these candidates appear functionally relevant, additional expression studies and further genetic analyses are required to provide additional evidence for their role in intumescence susceptibility.

Past studies have reported negative impacts of leaf intumescence development, including reductions in photosynthesis, leaf necrosis, and senescence (Craver et al. 2014b; Roloff et al. 2004) in various taxa including *E. globulus* and *E. nitens* seedlings (Pinkard et al. 2006). The previous study in *Eucalyptus* (Pinkard et al. 2006) was focused on individual leaf responses as opposed to a whole plant response. At the whole plant level, we found no evidence that the transient outbreak of intumescence adversely affected the growth of our F₃ families, despite the severity of symptoms in some individuals. Rather, our results showed that at the family level, intumescence severity was positively correlated with total leaves, LMA, and height₁. Potential explanations for these findings may be either that larger plants were more affected by intumescence, or that plants that were more affected by intumescence subsequently grew taller and produced more leaves with higher LMA. Of relevance to the former hypothesis, Dungey et al. (1997) found positive genetic correlations between growth traits (height, DBH, and volume) and the severity of *Teratosphaeria* (previously *Mycosphaerella*) leaf disease in *E. globulus*, suggesting that the greater leaf area and abundance of new foliage in more rapidly growing trees provided a greater opportunity for leaf infection. This could similarly apply in our study, if for example a greater leaf area reduced aeration around larger plants with more leaves, increasing their susceptibility to intumescence development.

Alternatively, the second hypothesis may be true if intumescence development resulted in compensatory growth and aboveground resource allocation. For example, multiple studies have demonstrated that *E. globulus* is able to recover aboveground biomass following leaf damage events by remobilizing stored carbon to the growth of new shoots, up-regulating photosynthesis in the remaining healthy leaves, and reducing resource allocation to coarse root growth (Borzak et al. 2017; Eyles et al. 2009).

In forest trees, phenotyping for QTL analysis is generally conducted directly on the individuals used for linkage mapping in field trials (Freeman et al. 2009; Grattapaglia and Resende 2011). However, for traits such as disease resistance, this may not always be a practical option. For example, death of susceptible individuals could reduce the sample size available for phenotyping further traits. In addition, screening in controlled environments may be necessary when studying susceptibility to a disease not naturally present in a given field trial, or a disease that cannot be introduced due to quarantine controls (Butler et al. 2016). In this study, we performed QTL mapping in an outbred F₂ mapping population, using the family means of their OP F₃ families as phenotypic data. One of the potential problems with this technique is that the power of QTL detection may be reduced, or 'false' QTLs may be detected, if there is nonrandom outcrossing among F₂ trees that may dilute or override the genetic effects segregating in the F₂. Such nonrandom outcrossing may arise under open pollination through variation in the level of self-fertilization or the outcrossed pollen pool (Patterson et al. 2005). Such variation is possible given that genetic variation in both self-incompatibility (McGowen et al. 2010) and flowering time (Jones et al. 2011) have been reported in *E. globulus*. The variation in outcrossing rate is particularly an issue for growth-related traits that exhibit inbreeding depression in *E. globulus* (Costa e Silva et al. 2011; Hardner and Potts 1995). Quantitative genetic studies of growth traits in *E. globulus* have shown that the variation among OP families is uncorrelated with the additive genetic differences among the mother trees as estimated under outcrossing (Costa e Silva et al. 2010; Hodge et al. 1996; Volker 2002). However, such estimates are likely well correlated for traits exhibiting higher levels of narrow-sense heritability and that are less affected by inbreeding depression (Potts and Vaillancourt 2004). Indeed, significant positive correlations have been shown for the latter traits, including wood density (Volker 2002), disease susceptibility (Dungey et al. 1997), as well as vegetative and reproductive phase change (Jordan et al. 1999). Thus, for such traits, we would expect that under an assumption of a random mating, the average performance of the open-pollinated progeny of an F₂ female tree would well reflect the general combining ability (i.e., half the breeding value) of the F₂ female, and provide robust data for mapping underlying QTLs. Such data would be expected to be free of environmental effects impacting the phenotype of the F₂ females (Lander and Botstein 1989).

In order to test the reliability of our approach, we scored phase change in the OP F₃, a trait previously scored in the F₂ mapping population (Hudson et al. 2014). Hudson et al. (2014) detected several QTLs influencing phase change in this F₂, including one major QTL with biparental segregation (meaning that the QTL was heterozygous in both parents), suggesting this QTL was likely to be associated with population-level differentiation between the two ecotypes. The major QTL we detected in the OP F₃ (*QPC1*) peaked at the same location and shared the same biparental inheritance as the major QTL detected in the F₂ (Hudson et al. 2014), providing strong evidence it reflected the same underlying locus (or loci). The second, less significant QTL detected with the OP F₃ data (*QPC2*; Table 2) was not reported in Hudson et al. (2014), but an examination of the LOD profiles in the F₂, using data from Hudson et al. (2014), found a QTL peak at this location below the suggestive significance level (chromosome-wide type I error rate <0.05). We did not detect four minor QTLs for vegetative phase change reported from analysis of the F₂ (Hudson et al. 2014). Rather than reduced power of the progeny testing approach per se, this likely reflects (i) the different measurements (presence/absence of phase change versus the node to vegetative phase change) used to assess phase change,

as well as (ii) our phenotypic data representing fewer F₂ individuals. Therefore, by providing evidence that QTLs found in the F₂ can also be found in the OP F₃, we support the validity of phenotyping using open-pollinated families from a mapping population. With appropriate experimental design measures (i.e., a large number of replicates), the progeny testing approach offers the advantages of prolonging the life of forest tree mapping populations by allowing the study of juvenile-expressed traits, genotype by environment interactions, as well as studies on disease resistance, which are required to be conducted in controlled environments due to quarantine laws. The same approach could also be used to extend the use of populations developed for association genetics.

In conclusion, our results demonstrate that the variability in intumescence severity in glasshouse-grown *E. globulus* has a significant genetic basis. The QTLs and candidate genes identified in this study provide a robust framework for further physiological and genetic studies, which would potentially elucidate underlying mechanisms and loci responsible for intumescence formation. There was only one case of colocation of a QTL for intumescence severity with our previously identified disease resistance QTL; arguing the genetic mechanisms are largely independent, and that pathogen damage and intumescence formation have not been confounded in our previous studies of resistance to the pathogens in this study. Similarly, under the experimental conditions tested where the outbreak was subsequently controlled, no adverse relationship between intumescence severity and growth at the whole-plant level were detected. In addition, the detection of the same major QTL affecting vegetative phase change as found by Hudson et al. (2014) supports using open-pollinated families for the phenotyping of parental mapping populations for future genetic studies, particularly for traits that are not impacted by inbreeding depression. This is likely to apply to many diseases, and the progeny testing strategy would allow for (i) mapping QTLs for multiple diseases in diverse environments without compromising the health of the plants used to generate the linkage map, and (ii) mapping QTLs for juvenile-expressed traits using a mature mapping population.

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