



# Genetic analysis of callus formation in a diversity panel of 96 rose genotypes

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## Abstract

In a diversity panel of 96 rose genotypes, variation in the capacity to form calluses on leaf explants *in vitro* was investigated, and a genome-wide association study (GWAS) was performed to identify genetic factors associated with callus formation. Calluses were induced from wounded *in vitro* leaflets on two media differing in their plant growth regulator composition. Significant differences between genotypes were observed in callus size on the first callus-inducing medium (CIM1, containing 10.7  $\mu\text{M}$  naphthylene acetic acid) using a 0–4 scale, as well as on a second callus-inducing medium (CIM2, containing 4.5  $\mu\text{M}$  dichlorophenoxyacetic acid and 2  $\mu\text{M}$  6-( $\gamma,\gamma$ -dimethylallylaminopurine)) with callus size scales of 0.82–4. GWAS utilizing the WagRhSNP 68K SNP array for callus size induced on either CIM1 or CIM2 enabled the identification of 26 and 13 significantly associated SNPs, respectively. Among these SNPs, we found the SNPs Rh12GR\_12098\_1092Q (uncharacterized gene) and RhMCRND\_2903\_1233Q in a gene encoding a pentatricopeptide repeat-containing protein were associated with callus size on CIM1, with large effects being observed between alleles. Two SNPs, RhK5\_5473\_763P (*S*-formylglutathione hydrolase) and Rh12GR\_37799\_568Q (polyglutamine binding protein, WW domain binding protein), were associated with callus size on CIM2 with large effect sizes. The markers associated with callus size on CIM1 form a large cluster on chromosome 3 and minor clusters on other chromosomes and provide the first preliminary indications of candidate genes responsible for the observed phenotypic variation.

## Key message

Callus formation in rose is strongly genotype dependent and varies continuously among 96 genotypes. A major QTL associated to callus size was located on chromosome 3.

**Keywords** *Rosa x hybrida* · Callus induction · SNP markers · Genome-wide association study (GWAS)

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## Introduction

Roses are among the most popular and economically important horticultural crops. These plants are used for many different purposes, such as ornamental plants as cut flowers, potted plants and garden plants, as well as for the food, pharmaceutical and perfume industries (Leus et al. 2018). Currently, there are approximately 30,000–35,000 known cultivated rose varieties, most of which are tetraploids of complex interspecific hybrid origin, are highly heterozygous and cover a wide phenotypic variability (Bendahmane et al. 2013; Kirov et al. 2014). However, roses propagated by seeds will not fall true-to-type, vegetative propagation by cuttings, layering, budding and grafting may be time-consuming, and there may be a limitation in stock plants (Marchant et al. 1996). *In vitro* propagation of roses allows

rapid multiplication, the production of disease-free plants and the application of genetic engineering to test gene functions and accelerate breeding programs. However, the high input of labor and strong genotypic differences in propagation and rooting efficiency make the *in vitro* propagation of roses economically infeasible for most genotypes.

When conducting genetic engineering, *in vitro* regeneration is a prerequisite, and regeneration via organogenesis and somatic embryogenesis often involves the induction of calluses, e.g., undifferentiated and proliferating cells, as the first step. Furthermore, callus formation is important to seal wounds, prevent water loss and provide cellular sources for vasculature differentiation (Ikeuchi et al. 2017). Incubating various types of explants on auxin-rich callus-inducing media (CIM) can induce callus formation (Dale and Deambrogio 1979; Ikeuchi et al. 2013; Bello et al. 2018; Shin and Seo 2018). Callus induction occurs when plant cells dedifferentiate and proliferate. This process is controlled by many factors, particularly the interplay of the plant hormones auxin and cytokinin, and it requires the expression of developmental genetic factors, such as *PASTICCINO* (*PAS*) genes, for coordinating cell division and differentiation of plant cells during development (Harrar et al. 2003). A rapidly transmitted wound signal activates jasmonic acid (JA) and its biologically active derivatives, which regulate a wide range of biological processes, including plant defense, secondary metabolism and growth (Koo et al. 2009). During callus development, many upregulated genes are involved in the response to stress, and callus development displays histological features similar to the root meristem, which is controlled by the spatial expression of root meristem regulator genes, such as *WOX5* and *SHORT ROOT* (Sugimoto et al. 2010). Similar to root formation, callus formation was shown to be activated by the expression of transcription factors, such as *LATERAL ORGAN BOUNDARIES DOMAIN (LBD)16*, *LBD17*, *LBD18*, and *LBD29* (Fan et al. 2012; Kareem et al. 2015; Okushima et al. 2007). The *ARABIDOPSIS TRITHORAXRELATED 2* (*ATXR2*) was employed to *LBD* promoters by the transcription factors *AUXIN RESPONSE FACTOR 7* (*ARF7*) and *ARF19* and regulated callus formation (Lee et al. 2017). Another factor influencing callus formation is the gene *ENHANCER OF SHOOT REGENERATION1*, which was shown to be directly upregulated by *WOUND INDUCED DEDIFFERENTIATION1*, an *Apetala 2/ethylene response factor* transcription factor (*AP2/ERF*) in *Arabidopsis thaliana* that stimulates callus formation and shoot regeneration (Che et al. 2006; Iwase et al. 2011, 2017). The reactivation of core cell cycle regulators, such as *CYCLIN* (*CYC*) and *CYCLIN-DEPENDENT KINASES* (*CDK*), leads to callus formation and organ regeneration. (Cheng et al. 2015; Inzé and Veylder 2006). Furthermore, very-long-chain fatty acid synthesis is catalyzed by the enzyme *3-KETOACYL-COA SYNTHASE 1* (*KCS1*),

whose mutation enhances callus formation from pericycle cells (Shang et al. 2016), and the genes *ETHYLENE RESPONSE FACTOR 115*, *PLETHORA3*, *PLETHORA5* and *PLETHORA7* have been recently identified as factors involved in callus generation (Sena et al. 2009; Xu et al. 2018). Although these physiological and molecular studies identified genes with effects on callus induction in recent years, the molecular mechanisms and the integration of environmental and endogenous signals are highly complex and have not been fully elucidated to date.

Several studies on callus induction in roses have been performed, mostly for developing transformation and/or regeneration protocols (Canli 2003; Hsia and Korban 1996; Huang et al. 2018; Khosh-Khui and Sink 1982; Kuusiene and Kandzeauskaite 2001; Noriega and Söndahl 1991; Pati et al. 2010; Zakizadeh et al. 2010). However, neither the genetic complexity of callus formation of roses nor the genes involved in these processes have been studied to date. In recent years, genome-wide association studies (GWASs) have been found to be an effective strategy for discovering underlying complex genetic traits (Chen et al. 2017). In roses, GWAS has been used to determine the loci associated with anthocyanin and carotenoid concentrations in petals (Schulz et al. 2016) with adventitious shoot and root regeneration (Nguyen et al. 2017, 2020) and petal numbers (Hibrand Saint-Oyant et al. 2018). These studies are the first examples for identifying quantitative trait loci (QTLs) via GWAS and the discovery of genes and markers for complex traits of roses.

In this study, we investigated the capacity for callus induction of 96 rose genotypes in a diversity panel previously analyzed for other traits (Nguyen et al. 2017; Schulz et al. 2016). Based on SNPs from the Axiom WagRhSNP array (Koning-Boucoiran et al. 2015), the variation in callus induction of 96 rose genotypes was analyzed using GWAS. The aim of this study was to identify SNP markers and chromosome (ChR) regions, as well as candidate genes, associated with the callus formation ability of rose.

## Materials and methods

### Plant material and *in vitro* establishment

The nodal stem segments of 96 rose genotypes (Supplementary Table S1) close to the apical meristem were collected from healthy plants in the greenhouse of the Federal Plant Variety Office in Hannover, Germany. The population was chosen because other traits had been analyzed previously and all plants had already been genotyped with an SNP array (Nguyen et al. 2017; Schulz et al. 2016). The stem segments were surface disinfected for 1 min in 70% ethanol, then for 10 min in 1% sodium hypochlorite solution and

finally rinsed 4 times in sterile deionized water. The culture medium for shoot proliferation consisted of MS (Murashige and Skoog 1962) basal salts with ferric ethylenediamine di-2-hydroxyphenyl acetate (instead of ferric ethylenediamine tetraacetic acid), 30 g L<sup>-1</sup> sucrose, 8 g L<sup>-1</sup> plant agar, 2.22 μM BAP (benzylaminopurine) and 0.58 μM GA<sub>3</sub> (gibberellic acid) (Duchefa, Harlem, Netherlands) as described in Nguyen et al. (2017). After two weeks, the shoots emerging from the axillary buds were excised and transferred to fresh medium to promote shoot growth and proliferation.

## Callus induction

Leaves of the upper part of vigorously growing *in vitro* shoots were used to prepare explants for callus induction. The petioles of single leaflets were removed, and three incisions were incised on the abaxial surface of the leaflet. All leaflet explants were placed with the adaxial surface in contact with the medium. Two media, CIM1 and CIM2 (Table 1), that had been used previously to induce embryogenic calluses in roses and cyclamen (Dohm et al. 2001; Prange et al. 2010), were compared.

For each rose genotype, ten leaflet explants were cultured in Petri dishes with 94 mm diameter with five replicates each, and the experiment was repeated three times. The explants were incubated in darkness for four weeks at 24 ± 2 °C. Callus development was scored based on the proportion of callus covering the leaflet using a 0–4 scale (Fig. 1), where 0 indicated no callus formation, 1 indicated less than 25% of the leaflet covered by callus, 2 represented 2–50% coverage, 3 indicated 51–75% coverage, and 4 signified more than 75% of the leaflet being covered by callus (Tuskan et al.

2018). The average callus size per experiment was calculated as.

Callus size =  $n \times G/N$  with  $n$  as the number of explants forming callus,  $G$  as the scale of callus rating for each explant and  $N$  being the total number of explants.

## Statistical analyses

Data were analyzed for differences between genotypes and repetitions of the experiments with the Kruskal–Wallis test. Normal distribution of the traits was tested using a quasibinomial model. The correlation coefficient between callus traits was calculated with Pearson's rank correlation. All statistical analyses were performed with the R software package, version 3.2.5 (The R-foundation for statistical computing 2016).

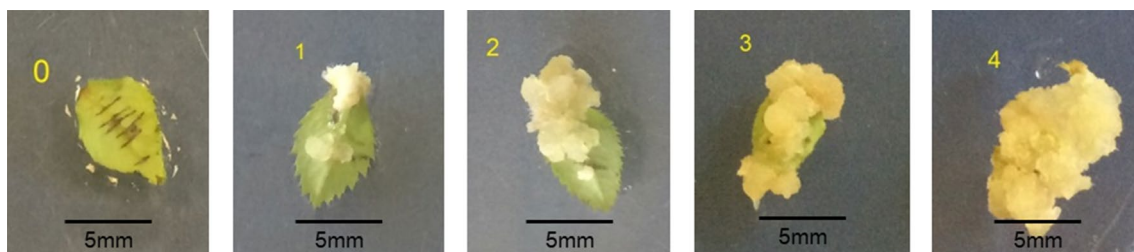
## Association mapping

SNPs were analyzed with the Axiom WagRhSNP 68K chip, which comprises 68,893 SNPs (scored on both DNA strands independently) derived from cut and garden roses (Koning-Boucoiran et al. 2015). The SNP dosage was estimated for each of the five allelic classes by fit Tetra (AAAA, AAAB, AABB, ABBB and BBBB) (Voorrips et al. 2011).

The association analysis was performed in TASSEL, version 3.0 (Bradbury et al. 2007) using information from the 96 genotypes for callus induction and genotypic data comprising 68,893 SNPs. To investigate associations between SNPs and callus formation traits, a linear mixed model was used with a minor allele frequency of 0.05. The Q matrix was obtained using STRUCTURE, version 2.3 (Hubisz et al. 2009) based on a subset of markers. The K matrix was

**Table 1** Composition of callus induction media CIM1 and CIM2

Media	Salts and vitamins	Plant growth regulators (μM)	Carbon source	Solidifying agent
CIM1	Full-strength MS basal salts and vitamins	NAA (10.7)	30 g L <sup>-1</sup> glucose	4.0 g L <sup>-1</sup> Gelrite
CIM2	Full-strength MS basal salts and vitamins	2.4D (4.5) 2iP (2)	30 g L <sup>-1</sup> glucose	4.0 g L <sup>-1</sup> Gelrite



**Fig. 1** Example of the rating of callus size with relative visual scores between 0 and 4. The rating of callus size is given by the numbers at the top of each picture with 1 indicating less than 25%; 2 = 26–50%; 3 = 51–75%; and 4 more than 75% of the leaflet being covered by callus

calculated with SPAGeDi 1.3 software (Hardy and Vekemans 2002). Association analyses were performed for each trait. The significance between traits and markers in the association was defined with the Bonferroni method using a threshold set to  $-\log p_{10} > 6.7$  ( $< 3.3E-06$ ). The allelic class effects were obtained directly from the TASSEL output.

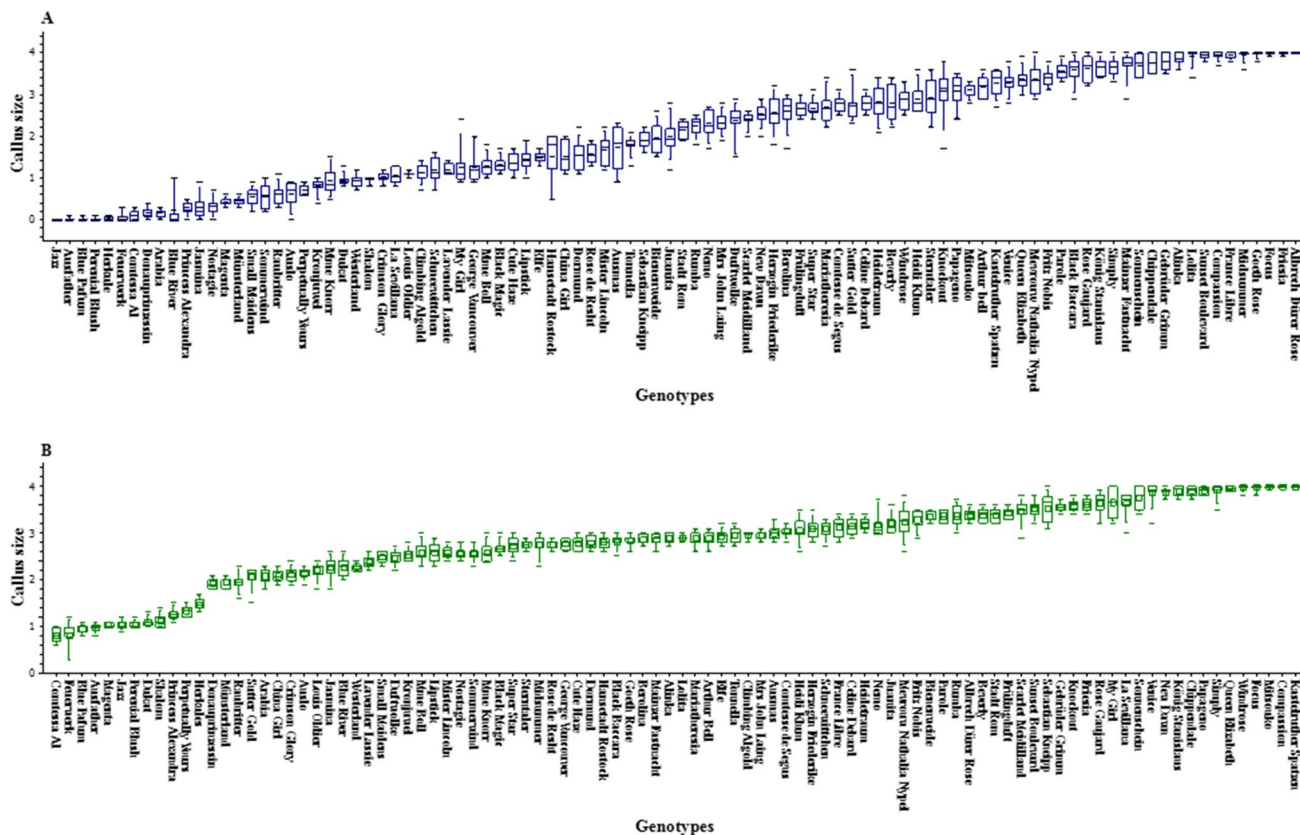
To visualize the associations, significant SNPs were used to blast against the *Rosa chinensis* ‘Old Blush’ genome (Hibrand Saint-Oyant et al. 2018) for localized SNP searching in the rose ChR from Bio Edit (Hall 1999). A homology search via a BLAST analysis on <https://blast.ncbi.nlm.nih.gov/Blast.cgi> was performed to locate the genes associated with the traits.

## Results

### Callus formation

Callus formation started from the edges of wounds of the leaflet explants and gradually grew to completely cover the explants after 28 days in the case of some genotypes

(Fig. 1, Fig. S1). The amount of callus, expressed on a callus scale of 0 to 4, varied considerably among genotypes (Supplementary Table S1, Fig. S1). On CIM1, 95 of 96 genotypes showed callus formation, with only leaflets of the cultivar Jazz failing to form callus (Fig. 2a). On CIM2, leaflets of all genotypes formed calluses, with callus sizes ranging from 0.8 to 4 (Fig. 2b). Overall, the sizes were higher than those recorded on CIM1. Interestingly, on both media, the lowest callus size was observed for the same group of genotypes, including Jazz, Ausfather, Blue Perfume, Perennial Blush, Comtessa AI, Feuerwerk, Magenta and Herkules (Fig. 2). Statistical analysis of the data for callus induction on both CIM1 and CIM2 revealed significant differences between genotypes at  $p = 0.05$  using a Kruskal–Wallis test, whereas no significant differences were revealed between the repeat experiments (Tukey’s test). In addition to callus formation, other *in vitro* regeneration traits that had been analyzed in previous studies (Nguyen et al. 2017, 2020) were used to analyze pairwise correlations (Table 2). A high correlation was found between callus size on CIM1 and CIM2 (0.76), whereas weaker but highly significant correlations were observed between the shoot propagation rate and callus size (CIM1:



**Fig. 2** Average callus size of the 96 rose genotypes after four weeks of culture on CIM1 (a) and CIM2 (b) based on three independent experiments using five biological replicates (with ten explants each).

Small square = mean; horizontal lines = median; minimum, maximum; box = 1st and 3rd quartiles; and whisker = standard deviation

**Table 2** Pearson's correlation coefficients for different *in vitro* traits of 96 rose genotypes (from the present and previous studies) and the corresponding p-values (in italics)

Pearson's correlation coefficient	Callus induction CIM2	Axillary shoot <sup>c</sup> proliferation	Adventitious shoot regeneration <sup>a</sup>	Root number <sup>b</sup>	Root length <sup>b</sup>
Callus induction CIM1	<b>0.76</b> <i>&lt; 2.2 *10<sup>-16</sup></i>	<b>0.54</b> <i>1.50 *10<sup>-8</sup></i>	<b>0.29</b> <i>0.005</i>	<b>0.37</b> <i>1.83 *10<sup>-3</sup></i>	<b>0.21</b> <i>0.039</i>
Callus induction CIM2		<b>0.63</b> <i>7.60 *10<sup>-12</sup></i>	<b>0.26</b> <i>0.010</i>	<b>0.43</b> <i>1.24 *10<sup>-5</sup></i>	<b>0.27</b> <i>0.007</i>
Axillary shoot proliferation <sup>c</sup>			0.06 <i>0.56</i>	<b>0.59</b> <i>4.45 *10<sup>-10</sup></i>	<b>0.34</b> <i>6.68 *10<sup>-3</sup></i>
Adventitious shoot regeneration <sup>a</sup>				0.04 <i>0.68</i>	0.00 <i>0.98</i>
Root number <sup>b</sup>					<b>0.70</b> <i>1.85 *10<sup>-15</sup></i>

Significant correlations are given in bold

<sup>a</sup>Data published by Nguyen et al. (2017)

<sup>b</sup>Data published by Nguyen et al. (2020)

<sup>c</sup>Unpublished data

0.54 and CIM2: 0.63) and with adventitious shoot regeneration and *in vitro* rooting traits (Table 2).

### Marker-trait association analysis

GWAS was performed with the data for the average callus size of the 96 rose genotypes to identify and localize genetic factors associated with this trait. For callus induction on CIM1, 21 SNPs significantly associated with callus size were found (Table 3; Fig. 3a). Almost all SNPs colocalized on Chr 03 and formed one large conspicuous cluster, indicating a major effect QTL at this position in the rose genome. Only three significant SNPs were found on Chr 00, forming a second cluster. Some SNPs had large effects, such as Rh12GR\_12098\_1092Q at position 370111 on Chr 03, Rh12GR\_6077\_815P at position 5193454, Rh12GR\_86832\_276 and RhMCRND\_2903\_1233Q at position 25447590 on Chr 03 (Table 3; Fig. 4). Apart from the significant p-value, these markers also display a dose-dependent effect of the alternative alleles (Fig. 4), further supporting the significance of the association between markers and traits.

GWAS analyses of the callus size on CIM2 revealed 11 significantly associated SNPs (Table 4; Fig. 3b). Among these SNPs, three were located on Chr02, six were on Chr03, one was on Chr04 and two were on Chr06. Some SNPs showed large effect sizes, such as Rh12GR\_37799\_568Q (NA) at position 6468674 on Chr 03 and RhK5\_5473\_763Q and RhK5\_5473\_763 at position 18402920 on Chr 03 (Table 4; Fig. 5). Of all the SNPs associated with callus size, only 2 SNPs

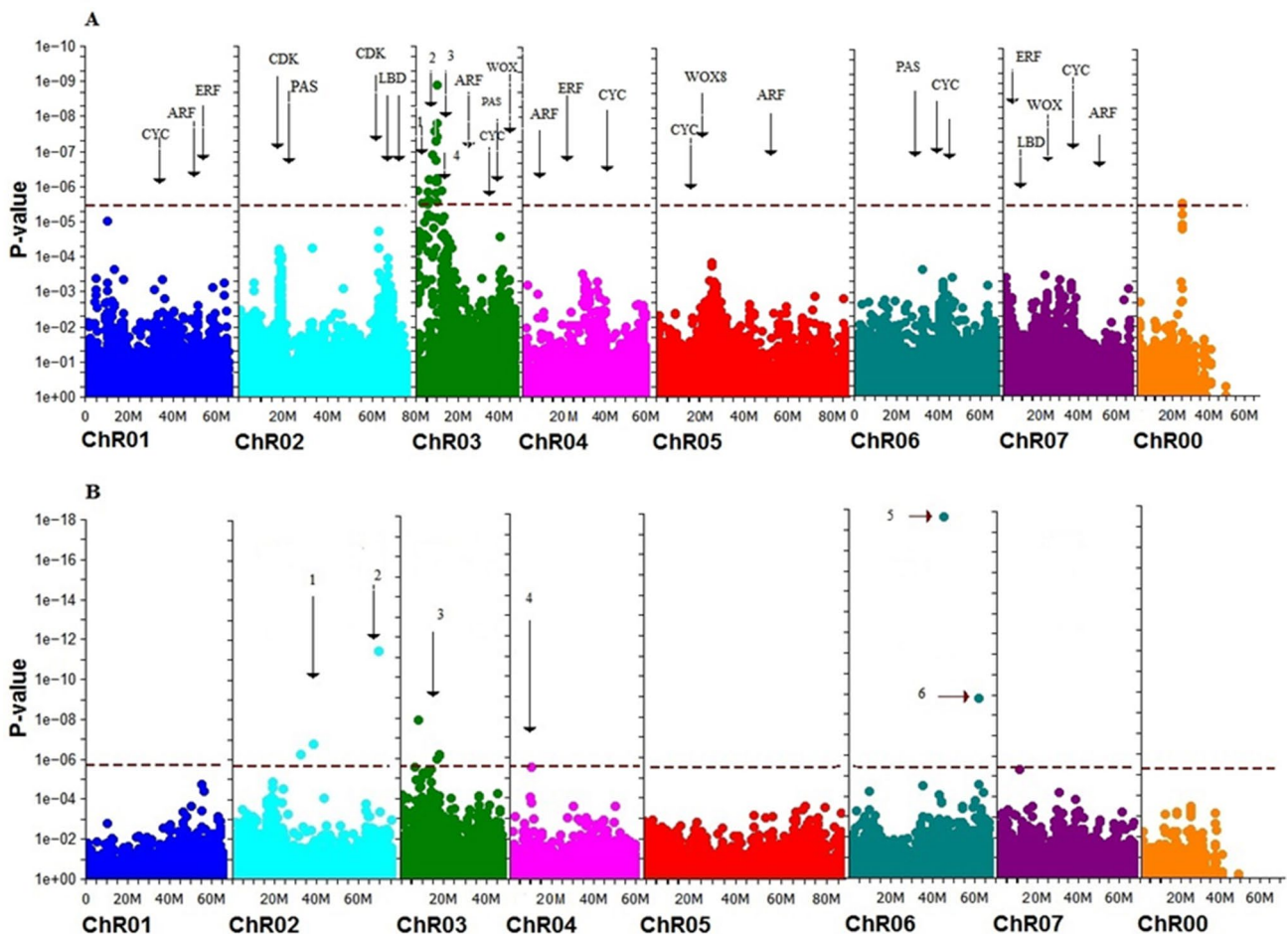
overlapped between CIM1 and CIM2. These SNPs were RhK5\_4750\_1179Q and Rh12RG\_37799\_568Q (Tables 3 and 4).

### Discussion

In this study, we present data on callus formation in an association panel comprising 96 rose genotypes on two *in vitro* media and its correlation to other traits related to developmental processes. Furthermore, we identified genomic regions associated with callus formation and located a selection of candidate genes possessing known functions for callus induction in relation to these regions.

### Callus induction in a panel of 96 rose genotypes

Callus induction is the first step for plant regeneration via somatic embryogenesis or via organogenesis for many plants, such as potato (Kumlay and Ercisli 2015) oil palm (Jayanthi et al. 2015; Yunista and Hapsoro 2011), bamboo (Yuan et al. 2013), *Lycium barbarum* (Osman et al. 2013), *Jatropha curcas* (Shamsiah et al. 2011), soybean (Yang et al. 2011), wheat (Ma et al. 2016) and hawthorn (Taimori et al. 2016). For roses, callus induction using leaf and stem explants was established first with *Rosa manetti* Hort. and *R. hybrida* L.cv. Tropicana (Khosh-Khui and Sink 1982). A range of different rose genotypes and plant hormones were used for callus induction. The callus of two cultivars, 'Heckenzauber' and 'Pariser Charme', was induced on CIM1 and gave rise to embryogenic cultures (Dohm et al. 2001). Our

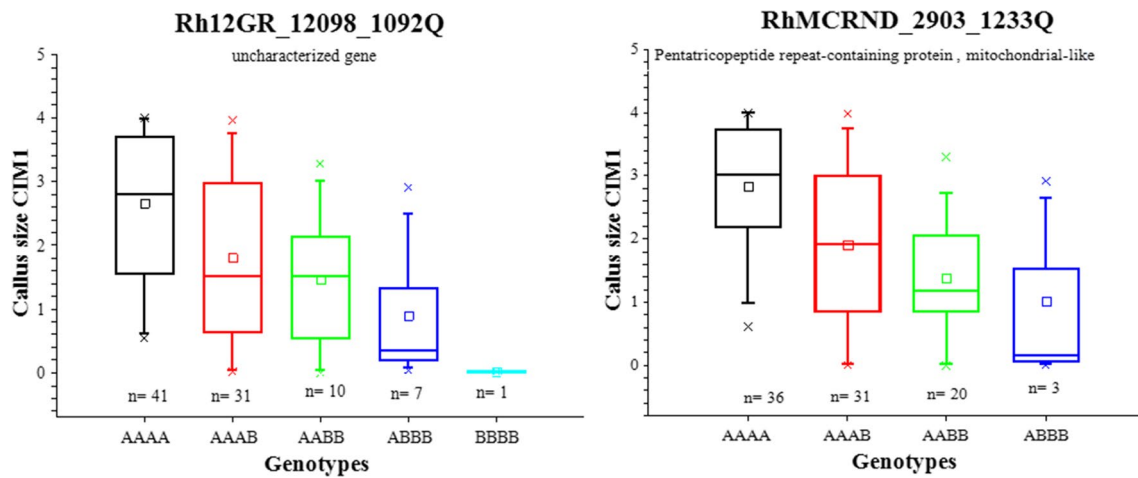


**Fig. 3** Manhattan plot of callus size induced on CIM1 (A) and CIM2 (B) The red dashed line represents the Bonferroni threshold of the adjusted significance level  $-\log_{10} p = 6.7$  The subdivision of the x-axis is by chromosome (Chr01–Chr00), including chromosome 00 with contigs not assigned to a precise location to date. Each scale bar of the x-axis represents 5 Mb. Note: **A** 1: *Rosa chinensis* spliceosome-associated protein 130 A, 2: *Rosa chinensis* protein C2-DOMAIN ABA-RELATED 5-like (LOC112192906), mRNA, 3: *Rosa chinensis* glutathione S-transferase DHAR3, chloroplastic (LOC112195020), mRNA, 4: *Rosa chinensis* putative pentatricopeptide repeat-containing protein At5g08490 (LOC112193021), transcript variant X2. Candidate genes: ARF auxin response factor, CYC cyclin, CDK cyclin dependent kinase, ERF ethylene-

responsive transcription factor, LBD LATERAL ORGAN BOUNDARIES, PAS PASTICCINO, WOX: WUSCHEL-related homeobox. **B** 1: *Rosa chinensis* transmembrane E3 ubiquitin-protein ligase 1 (LOC112188470), transcript variant X1, mRNA, 2: *Rosa chinensis* uncharacterized CRM domain-containing protein At3g25440, chloroplastic (LOC112193599), transcript variant X1, mRNA, 3: *Rosa chinensis* polyglutamine binding protein, WW domain binding protein, 4: *Rosa chinensis* protein SULFUR DEFICIENCY-INDUCED 2 (LOC112201022), mRNA, 5: *Rosa chinensis* chromatin modification-related protein EAF1 B-like (LOC112172241), transcript variant X2, mRNA, 6: *Rosa chinensis* 54S ribosomal protein L24, mitochondrial (LOC112174756), mRNA

comprehensive data set enables a detailed comparison of callus formation in two different media among 96 genotypes, the largest number of rose genotypes in a single study on callus formation published to date. Our data indicated that on CIM2, explants of rose genotypes on average formed more calluses than on CIM1, but the genotypes with a small average callus size were similar on both media. This observation and a high correlation of callus formation between the two media (Table 2) suggested that they were controlled at least in part by the same genetic factors. This finding is expected, as both media used auxin as the major growth regulator

and only differed in the type of auxin (NAA in CIM1 and 2,4-D in CIM2), as well as an additional cytokinin (2iP) in CIM2. Although we found a lower correlation between callus size and other *in vitro* traits, some of these correlations, such as axillary shoot proliferation, ranged between 0.54 and 0.63 for CIM1 and CIM2, respectively, and were highly significant (Table 2). It is tempting to speculate that common genetic factors play a role in both processes, and likely candidates might be found in the role of auxin perception and signaling, which are crucial for both callus formation and the outgrowth of side shoots in plants. This finding might



**Fig. 4** Genotypic effects of SNP markers associated with callus size on CIM1, Rh12GR\_12098\_1092Q (*Rosa chinensis* uncharacterized LOC112192505) and RhMCRND\_2903\_1233Q (*Rosa chinensis* pentatricopeptide repeat-containing protein At5g15010,

mitochondrial-like (LOC112192673)). Small square = mean; continuous line = median; asterisk = minimum, maximum; box = 1st and 3rd quartiles; and whisker = standard deviation)

also be observed for the somewhat weaker but still highly significant correlations of callus size to root numbers (0.37 and 0.43). Some authors have found that signaling networks between callus of *Arabidopsis* induced on callus-inducing media partially overlap with those leading to the formation of lateral root primordia, which might cause some of the correlations (Sugimoto et al. 2010; Fan et al. 2012; Ikeuchi et al. 2013). However, based on these data alone, causal relations cannot be inferred and need further functional analyses by, for example, overexpression or knockdown/knockout studies involving candidate genes identified for callus induction.

### Marker-trait association analysis

Recently, marker-trait associations have been analyzed for callus induction in a number of plants, such as *Populus trichocarpa* (Tuskan et al. 2018), soybean (Yang et al. 2011), rice (Zhang et al. 2019) and maize (Ma et al. 2018). In the present study, we detected 21 SNPs associated with callus size after induction on CIM1 and 11 SNPs associated with callus size on CIM2. Apart from significant p-values of markers associated with a trait, the formation of a marker peak above markers constituting statistical background noise can be taken as an indication of genetic factors that influence the trait under investigation. Among the SNPs significantly associated with callus size, some deserve a more detailed discussion: the SNP Rh12GR\_59735\_1764Q is located in a gene encoding spliceosome-associated protein 130A. This gene belongs to alternative splicing factors that have roles in regulating gene expression during the development of multicellular organisms and are important for stress adaptation in plants (Staiger and Brown 2013). Moreover, the spliceosome-associated protein 130A plays an indispensable

role in the specific spatiotemporal events of reproduction (Aki et al. 2011). The SNPs Rh12GR\_13539\_496P and Rh12GR\_13539\_496Q are derived from genes encoding E3 ubiquitin-protein ligases similar to *ARKADIA*, which were found to be associated with the callus size induced on CIM1. RhK5\_12450\_841P lies in a gene encoding a *Rosa chinensis* transmembrane E3 ubiquitin-protein ligase 1 and is associated with the callus size induced on CIM2. These genes belong to the ubiquitination family and are involved in the regulation of cell cycle progression, transcriptional regulation, DNA repair, signal transduction and protein turnover (Ho et al. 2015; Pfeffer et al. 2015) and control organ size in a dosage-dependent manner in *Arabidopsis* (Disch et al. 2006).

For callus size on CIM2, we found an association with the SNP RhK5\_107\_2439P, which is located in a gene encoding a chromatin modification-related protein of the type EAF1 B-like (Fig. 3). This protein was identified in a stable subunit of *NuA4*, a complex of a yeast histone H4/H2A acetyltransferase implicated in DNA repair and gene regulation (Auger et al. 2008; Bieluszewski et al. 2015). The SNP RhK\_5473\_763P displays a strong effect between alleles associated with callus size on CIM2 (Fig. 5). This SNP is derived from a gene for S-formylglutathione hydrolase having a function in formaldehyde detoxification in animals and microorganisms, which was also found in *Arabidopsis* (Kordic et al. 2002). The S-formylglutathione hydrolase was suggested to be involved in the detoxification of xenobiotics, such as herbicides (Gershater and Edwards 2007). It can be speculated that this enzyme might be involved in the metabolism of 2,4-D that was added to our medium CIM2. The gene for the 54S ribosomal protein L24, which harbors marker RhMCRND\_10042\_489P, was associated

**Table 3** Significant SNPs associated with callus size induced on CIM1

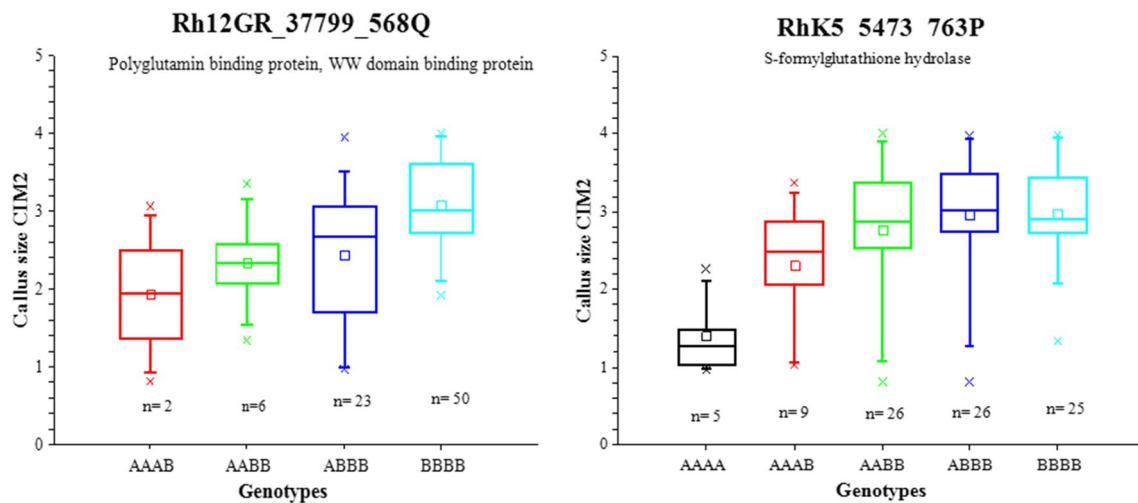
Marker	p-value	Effects			Chr	Position	Gene
		A:A	A:B	B:B			
Rh12GR_27683_2069P	1.21E-09	–	0	1.48	3	10166387	<i>Rosa chinensis</i> probable fructokinase-6, chloroplastic (LOC112194730)
Rh12GR_4846_920P	2.62E-08	–	0	1.36	3	8790885	<i>Rosa chinensis</i> DEAD-box ATP-dependent RNA helicase 13 (LOC112193330)
Rh12GR_59753_1764Q	3.63E-08	–	0	1.43	NA	NA	<i>Rosa chinensis</i> TATA_element_modulatory_factor_(TMF)
Rh12GR_25423_3834P	4.84E-08	–	1.30	0	3	9153717	<i>Rosa chinensis</i> spliceosome-associated protein 130 A
RhK5_4750_1179Q	1.20E-07	1.58	0	–	3	7868346	<i>Rosa chinensis</i> uncharacterized CRM domain-containing protein At3g25440, chloroplastic (LOC112193599), transcript variant X2
Rh12GR_13539_496P	1.62E-07	–	– 1.24	0	0	2687062	<i>Vitis vinifera</i> E3 ubiquitin-protein ligase Arkadia (LOC100248215)
RhMCRND_13074_681P	5.45E-07	1.36	0	–	3	9613831	<i>Rosa chinensis</i> protein C2-DOMAIN ABA-RELATED 5-like (LOC112192906)
RhMCRND_9915_389Q	5.69E-07	0	–	– 1.23	3	9758183	<i>Rosa chinensis</i> glutathione S-transferase DHAR3, chloroplastic (LOC112195020)
Rh12GR_59259_108P	6.19E-07	2.05	0.89	0	3		<i>Rosa chinensis</i> BTB/POZ and MATH domain containing protein
RhMCRND_9892_919P	7.11E-07	–	– 1.25	0	3	9165684	<i>Rosa chinensis</i> uncharacterized LOC112193027 (LOC112193027), transcript variant X1, mRNA
Rh12GR_12098_1092Q	1.30E-06	–	– 1.23	0	3	370111	<i>Rosa chinensis</i> uncharacterized LOC112192505, transcript variant X4
RhK5_6755_333P	1.33E-06	–	0	1.16	3	11931437	<i>Rosa chinensis</i> transcription termination factor MTERF4, chloroplastic (LOC112193459)
Rh12GR_6077_815P	1.37E-06	0	– 1.18	–	3	5193454	<i>Rosa chinensis</i> probable lysophospholipase BODYGUARD 4 (LOC112192624), transcript variant X1
RhMCRND_11099_934P	1.50E-06	–	– 1.15	0	3	9179620	<i>Rosa chinensis</i> psbP domain-containing protein 6, chloroplastic (LOC112191588), transcript variant X1
Rh12GR_37799_568Q	2.50E-06	0	– 1.18	–	3	6468674	<i>Rosa chinensis</i> polyglutamine binding protein, WW domain binding protein
RhMCRND_20513_1468P	2.51E-06	0	– 1.18	–	3	5667332	<i>Rosa chinensis</i> putative pentatricopeptide repeat-containing protein At5g08490 (LOC112193021), transcript variant X2
Rh12GR_19029_1911P	2.60E-06	–	– 1.20	0	0	25447725	<i>Rosa chinensis</i> pentatricopeptide repeat-containing protein At5g15010, mitochondrial-like (LOC112192673), transcript variant X1
Rh12GR_86832_276P	2.67E-06	0	– 1.16	–	NA	NA	<i>Rosa chinensis</i> U-box_domain-containing_protein_4
Rh12GR_81252_184Q	2.80E-06	0	– 1.16	–	NA	NA	<i>Rosa chinensis</i> polyglutamine binding protein, WW domain binding protein
RhMCRND_2903_1233Q	3.00E-06	–	0	– 1.14	0	2544759	<i>Rosa chinensis</i> pentatricopeptide repeat-containing protein At5g15010, mitochondrial-like (LOC112192673), transcript variant X1
Rh12GR_54251_670P	3.05E-06	0	– 1.15	–	3	5665174	<i>Rosa chinensis</i> putative pentatricopeptide repeat-containing protein t5g08490(LOC112193021), transcript variant X2

with callus size on CIM2 and has a function in controlling developmental programs through translational regulation of auxin response factors (Rosado et al. 2012).

In addition to the markers significantly associated with callus induction on CIM1, we found a clear formation of peaks of marker clusters. In this instance, the largest number of significant SNPs and the strongest clustering of markers leads to a conspicuous broad peak of markers at the beginning of chromosome 3. This cluster spanning

a large region of more than 15 Mb does not comprise any of the most prominent genes influencing callus induction, e.g., *WOX*, *ARF*, *CDK* or *CYC* genes. However, the predicted transcripts of the reference genome of *R. chinensis* cv. Old Blush (Hibrand Saint-Oyant et al. 2018) for this region indicates that this region harbors several predicted genes with similarity to factors also known to be either directly involved in either callus formation or in related developmental processes. Examples of such genes are





**Fig. 5** Genotypic effects of SNP markers associated with callus size on CIM2, RhK5\_5473\_763P (*Rosa chinensis* S-formylglutathione hydrolase (LOC112191850), mRNA and Rh12GR\_37799\_568Q (*Rosa chinensis* polyglutamine binding protein, WW domain bind-

ing protein). Small square = mean; continuous line = median; asterisk = minimum, maximum; box = 1st and 3rd quartiles; and whisker = standard deviation)

**Table 4** Significant SNPs associated with callus size induced on CIM2

Marker	p-value	Effects			ChR	Position	Gene prediction
		A:A	A:B	B:B			
RhK5_107_2439P	2.24E-18	-	0	0.51	6	45395443	<i>Rosa chinensis</i> chromatin modification-related protein EAF1 B-like (LOC112172241), transcript variant X2
RhMCRND_6130_146Q	3.2E-12	-	0.66	0	2	68676139	<i>Rosa chinensis</i> chorismate mutase 1, chloroplastic (LOC112188602)
RhMCRND_10042_489P	1.60E-09	0	-	0.51	6	62167206	<i>Rosa chinensis</i> 54S ribosomal protein L24, mitochondrial (LOC112174756)
RhK5_4750_1179Q	1.26E-08	1.14	0	-	3	7868346	<i>Rosa chinensis</i> uncharacterized CRM domain-containing protein At3g25440, chloroplastic (LOC112193599), transcript variant X1
RhK5_12450_841P	1.6E-07	0.62	0.29	0	2	38349478	<i>Rosa chinensis</i> transmembrane E3 ubiquitin-protein ligase 1 (LOC112188470), transcript variant X1
RhMCRND_4377_105P	5.3E-07	0	-1.63	-0.76	2	31990763	<i>Rosa chinensis</i> aspartic proteinase Asp1 (LOC112190217)
RhK5_5473_763P	6.12E-07	0.003	0	-1.68	3	18402920	<i>Rosa chinensis</i> S-formylglutathione hydrolase (LOC112191850)
RhK5_12078_99Q	1.00E-06	-1.71	0	-0.10	3	17475978	<i>Rosa chinensis</i> folylpolyglutamate synthase (LOC112194857), transcript variant X5
RhK5_6079_150Q	2.70E-06	-	1.23	0	4	9719228	<i>Rosa chinensis</i> protein SULFUR DEFICIENCY-INDUCED 2 (LOC112201022)
Rh12GR_37799_568Q	2.79E-06	-	-0.79	0	3	6468674	<i>Rosa chinensis</i> polyglutamine binding protein, WW domain binding protein
Rh12GR_3363_1266Q	3.20E-06	-	0	0.78	3	13761410	<i>Rosa chinensis</i> pectinesterase-like (LOC112191366)

*LOB* (LATERAL ORGAN BOUNDARIES) domain-containing factors at the position of 6 Mb, a *DELLA GAI-like* transcriptional regulator that acts as a repressor of the cytokinin pathway (Ito et al. 2018), a *PLATZ* (PLANT A/T RICH SEQUENCE AND ZINC BINDING PROTEIN) family transcriptional repressor involved in the regulation of cell division (Kim et al. 2018), a regulatory protein

of the *TORI* (TARGET OF RAPAMYCIN) type putatively modulating plant cell growth (Steiner et al. 2016), and *RSI-1* (REDUCED SYSTEMIC IMMUNITY), a molecular marker for auxin-induced lateral root initiation (Taylor and Scheuring 1994), as well as several LRR-receptor-like protein kinases and transcription factors. The relatively wide extension of this peak region on chromosome 3 might also

indicate the presence of more than one causal factor for callus induction.

At the second less defined peak at the end of chromosome 3, which does not reach the significance level, genes with similarity to *BABYBOOM* (*BBM1* and *BBM2*) involved in embryogenic callus formation (Ikeuchi et al. 2013; Yang et al. 2014; Du et al. 2019) are located at a position of approximately 46 Mb. More pronounced peaks were detected on chromosomes 0, two and five. Chromosome 0 comprises all sequences that could not be assigned unequivocally to a genomic position among the seven rose chromosomes. The first analysis indicates that Rcr0 comprises sequences from several chromosomes, including larger groups of contigs that might belong to chromosomes four and two, making it impossible to locate these sequences exactly (Hibrand Saint-Oyant et al. 2018). However, the clearly defined peak reaches the significance threshold and therefore comprises genetic factors with a strong influence on callus formation on CIM1. The two peaks on chromosome 2 comprise regions in which *CDK*, homologs of *PAS* genes and further *LBD* genes are located (Fig. 3a; Table 3), all of which are candidate genes that influence callus formation. Taken together, the data for callus induction on CIM1 indicate a major QTL on chromosome 3, as well as several minor QTLs on other chromosomes, including chromosomes 2, 3 and 5. The influence of other factors apart from the major QTL on chromosome 3 is also supported by the limited effect of markers derived from the region on chr. 3, indicating that despite the highly significant effect, they explain only part of the whole phenotypic variation.

In contrast to callus induction on CIM1, the induction on CIM2 containing the strong auxin 2,4-D did not lead to marker associations as clear as for CIM1. However, one region on chromosome 3 identical to the major QTL for callus induction on CIM1 is significant, and the minor regions on chromosome 2 also show some small peaks matching those for CIM1. In addition, a region in the lower region of chromosome 4, as well as on chromosome 6, shows minor peaks with individual significant markers. This finding indicates a tendency similar to callus induction on CIM1 with an overall smaller marker-trait association and further supports the hypothesis of common genetic factors discussed above for the correlation of both traits. The smaller effects of genomic regions on callus formation on CIM2 may be observed because more genes contribute to the phenotypic variation among the 96 plants tested. Other possible reasons are differences in the phenotypic variation between callus induction on CIM1 and CIM2 (Fig. 2): callus induction on CIM2 was more effective than on CIM1 in that all plants showed some degree of callus formation, and the average rates of callus induction were considerably higher on CIM2 than on CIM1 (Fig. 2), leading to a lower degree of differentiation among the analyzed genotypes. One can

speculate that an earlier time point for phenotyping callus development on CIM2 might have detected a stronger differentiation between genotypes and therefore would have uncovered stronger genetic effects at earlier time points than the time point actually used. Furthermore, we used only 96 genotypes for our GWAS analysis, which only enables the detection of QTLs with strong effects. Larger plant populations might reveal additional QTLs with smaller effects. For example, in wheat, QTLs for callus induction were identified on chromosomes 1D, 5A, and 6D (Ma et al. 2016) and on chromosomes B2 and D2 in soybean (Yang et al. 2011). In the *Populus* genome, eight loci were distributed across chromosomes III, IV, XI, VIII, IX, XII, XV and XVII (Tuskan et al. 2018). In rice, 21 significant loci located in rice callus induction QTLs were revealed (Zhang et al. 2019). Given the relatively small resolution, our GWAS population provided SNPs with significant association to the traits under study that might only be linked to the causal factors. These might not show up as an associated marker because the criteria for the design of the WagRhSNP chip were highly stringent, excluding many transcribed genes from marker design (Koning-Boucoiran et al. 2015). Therefore, further functional analyses are needed to identify the genes that led to the observed associations. Apart from analyzing larger plant populations, these studies might comprise the quantification of gene expression or in-depth sequence comparison for genotypes with contrasting capacity for callus formation, as well as transgenic experiments with either knockdown/knockout or overexpression of individual candidate genes and subsequent quantification of callus formation in vitro. However, the extent of such experiments was beyond the scope of the current study and they need to be addressed in subsequent investigations.

## Conclusions

In the present study, we showed that callus induction in roses is strongly dependent on both the genotype and the medium used. Though measurement of in vitro traits in large populations is extremely laborious, GWAS studies have the potential to unravel major genetic factors acting on those traits. By GWAS, we identified some genomic regions harboring factors with a strong genetic effect on callus formation with a major QTL located on chromosome 3. Among these SNPs, for callus size on medium CIM1 the SNPs Rh12GR\_12098\_1092Q (uncharacterized gene) and RhMCRND\_2903\_1233Q in a gene encoding a pentatricopeptide repeat-containing protein had large effects of 2.7 and 1.8, respectively. Furthermore, two SNPs, RhK5\_5473\_763P (S-formylglutathione hydrolase) and Rh12GR\_37799\_568Q (polyglutamine binding protein, WW domain binding

protein), were associated with callus size on CIM2 with large effect sizes (1.2 and 1.6, respectively). These markers with a strong association with callus formation can serve as a starting point for both the identification of genotypes with a strongly contrasting capacity for callus induction, which is useful for further studies on callus induction in roses, and for the identification of genes with effects on callus induction in roses, therefore extending our knowledge on developmental processes in this important woody ornamental.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflicts of interest.

**Informed consent** All authors declare their consent for this publication.

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