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Quantitative trait loci associated to drought tolerance in pea (*Pisum sativum* L.)

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KEY MESSAGE: Visual phenotyping as well as physiological parameters are considered together for the first time in a QTL analysis for drought tolerance, allowing the identification of 10 QTLs. Molecular markers related to these as well as candidate genes and their relationships with other maps from pea is discussed.

ABSTRACT: Pea (*Pisum sativum* L.) is an important grain legume whose yield stability and production is constrained by drought stress in most environments. Unfortunately, little is known on availability of drought tolerance sources and its genetic control in pea. In this work we assessed drought visual symptoms, and relative water content in soil (RWCS) and leaves (RWCL) along a time course of water stress on a pea Recombinant Inbred Lines (RILs) population from two parents known to segregate for drought tolerance. This allowed us to identify ten quantitative trait loci (QTLs) associated with the traits explaining individually from 9 to 33% of the phenotypic variation depending on the variable assessed and altogether from 20 to 57%. Molecular markers useful for Marker Assisted Selection and two candidate genes for drought tolerance were identified and the relation between the QTLs identified and other QTLs previously mapped for related traits is discussed.

Keywords: drought, water stress, relative water content, pea, QTL.

INTRODUCTION

Pea (*Pisum sativum* L.) is one of the most widely grown grain legumes in the world with primary production in temperate regions (Smýkal et al. 2012). One of its advantages as a legume crop relies on its capacity for symbiotic nitrogen fixation, allowing the reduction in the use of fertilizers in crop rotations. However, this process is highly sensitive to environmental stresses such as drought and salinity (Zahran 1999) which are major constraints to the production and yield stability of pea, especially during the flowering and pod filling (Doré et al. 1998).

Water deficit induces a range of physiological and biochemical responses within the plant which include stomatal closure, activation of respiration, repression of cell growth and photosynthesis. At the cellular level, plant responses to water deficit may result from cell damage, whereas other responses may correspond to adaptive processes. Dehydration in plant tissues induces changes in cell membrane stability and permeability, which finally lead to changes in the cell functions. Therefore, different mechanisms of response and physio-biochemical changes at both cellular and whole plant level are induced by drought, making of it a complex abiotic stress which tolerance is supposed to be controlled by different genomic regions (Shinozaki et al. 2007; Xoconostle-Cázares et al. 2011).

Visual assessment of drought symptoms evolution along a time course has been described as a fast approach to discriminate between tolerant and susceptible plants (Iglesias-García et al., 2012; Sánchez et al. 2012). The development of visual scales specifically adapted to a crop allows to indirectly assess a wide range of factors, not only the ability of the plants for water uptake and its preservation, but also others such as oxydative stress (by photoinhibition, lipid peroxidation, etc.) which could be yellowing the leaves.

As a complex trait it is, drought assessment would improve from its partitioning into components that are easy to measure such as relative water content (RWC) in soil (RWCS) and leaves (RWCL). Whereas RWCS has not been associated to tolerance or susceptibility to drought, it is a helpful parameter which allows indirect assessment of the water uptake by the plants. If RWCS decreases quickly, it could reflect a higher stress level within the plant than indicated by a slower decrease, but not necessarily lower plant efficiency under water stress conditions. Water use efficiency (WUE) by the plant is then strongly related with RWCS when tolerance and susceptibility to

drought is assessed, as if a plant shows a high water uptake but a low WUE it will have to deal with both these circumstances to survive during drought periods.

On the other hand, relative water content in leaves (RWCL) reflects the ability to maintain cell turgor when measured under drought stress, providing an idea of the tolerance capacity of the plant. Thereby, RWCL has been widely used as a physiological index in different crops for the evaluation of drought and temperature tolerance (Hunt et al. 1987; Tripathy et al. 2000; Siddique et al. 2000). The identification of the genes controlling RWC as well as molecular markers tightly linked to them by QTL mapping could be used as a tool to assist breeding for water stress tolerance. However, despite being a suitable index for water stress, the genes controlling RWC still remain unknown. No previous QTL analysis for drought tolerance have included this trait, probably because the measurement of this trait in large segregating populations can be tedious and expensive (Keurentjes et al. 2008).

The main objective of this work was to study the genetics of drought tolerance in pea and to identify molecular markers which can be useful to select tolerant genotypes in breeding programs. With this aim we assessed the evolution of drought symptoms and RWC in soil and leaves along a water stress time course in a Recombinant Inbred Line (RIL) population segregating for drought tolerance and performed a QTL analysis.

MATERIALS AND METHODS

Plant material and growth conditions

The population used in the study consisted of 98 F_{7:8} RILs families from a cross between P665 and cv. Messire. P665 (derived from the ICARDA accession IFPI3280) is a *Pisum sativum* subsp. *syriacum* full-leaved accession widely used in pea breeding due to its resistance to diseases (Fondevilla et al. 2005; 2010; Rubiales et al. 2009a, b) in spite of showing wild traits such as late flowering, creeping growth habit and violet flowers. Cv. Messire is a *P. sativum* subsp. *sativum* full-leaved, early-flowering and white-flowered pea cultivar. Parents P665 and cv. Messire were found as tolerant and moderately susceptible to water stress, respectively, in previous experiments (Iglesias-García et al. 2012).

Seeds from all the RILs families along with the parents were pre-germinated in Petri dishes with moistened filter papers in the dark for 48 h in a cold chamber at 4°C and then placed for another 48 h in a growth chamber at 65% relative humidity and 20°C. Seedlings were planted individually in 0,5 L pots filled with peat: sand (3:1) and placed into a growth chamber in a randomized complete block design with 9 replicates. Plants were grown at 21 °C, under a photons flux density (PPFD) of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by high output white fluorescent tubes.

Drought stress treatment

Plants were well watered during their 21 days growing period until the beginning of the drying episode, when plants were subjected to watering withdrawal. A minimum number of 4 plants per time point and line (RIL family or parent) were used for the measurements.

Visual scale assessment of water stress symptoms

From the beginning of the watering withdrawal all the RILs were assessed daily with a visual scale adapted to pea behavior from the one developed for oat (*Avena sativa*) by Sánchez-Martín et al. (2012). According to previous experiments (Iglesias-García et al. 2012), we used the 4th pair of leaves to evaluate drought symptoms uniformly on each line.

We assessed temporal evolution of water stress symptoms according to five stages, numbered from 1 to 4 (Online resource 1). Each status in the scale corresponds with the following characteristics: 1) No symptoms observed in the pair of leaves; 2) General softening of the pair of leaves; 3) Curved leaves with marked ribs; 4) Yellowing and/or border necrosis observed (0-50% of the pair of leaves).

Online resource 1. Visual scale of the representative symptoms. 1) No symptoms observed in the pair of leaves; 2) General softening of the pair of leaves; 3) Curved leaves with marked ribs; 4) Necrosis observed (0-50% of the pair of leaves).

Finally, data obtained were used to calculate the area under the drought symptoms progress curve (AUDPC) for each genotype, according to the equation: $AUDPC_VS = \sum [(x_i + x_{i+1})/2] * (t_{i+1} - t_i)$.

Where x_i = water stress symptoms at date i , x_{i+1} = of water stress symptoms at date $i+1$, and $t_{i+1} - t_i$ = number of days between scoring dates i and $i+1$. This trait was named “AUDPC_VS”.

Relative water content measurements

RWCS was calculated daily according to the methodology used by Bechtold et al. (2010). Briefly, pots were filled with identical amount of substrate and at the same time, three control pots were used to determine the weight of 100 and 0% soil water content, named saturated weight (SW) and dry weight (DW), respectively. Pot weight (FW) was determined daily and RWCS was calculated according to the formula $RWCS = (FW - DW) / (SW - DW)$. Pots were left to dry until less than 23% RWCS was reached in all the replicates.

RWCL was determined according to Cabrera-Bosquet et al. (2007). One leaflet of the third pair of leaves of each plant was collected for this measurement in accordance with RWCS assessment. Thus, samples were taken 0, 4, 9 and 15 days after watering withdrawal, corresponding to 100%, 75%, 45% and 22% of average RWCS, respectively. Leaf segments were weighed (fresh weight, FW), then saturated in water for 24 hours and their turgid weights (TW) were calculated. The samples were then dried in an oven at 60°C for 72 hours and weighed (DW). RWCL was determined as $RWCL = (FW - DW) / (TW - DW) \times 100$.

As both RWCS and RWCL were taken along a time course, we calculated the area under the drought progression curve (AUDPC_RWCS and AUDPC_RWCL, respectively) using the formula: $AUDPC_RWCS/L = \sum [(x_i + x_{i+1})/2] * (t_{i+1} - t_i)$.

Where x_i = estimated proportion of water content at date i , x_{i+1} = estimated proportion of water content at date $i+1$, and $t_{i+1} - t_i$ = number of days between scoring dates i and $i+1$. This trait represented the evolution of RWCS (AUDPC_RWCS) and RWCL (AUDPC_RWCL) along the time course for each RIL and the parents.

Statistical analysis and heritability estimation

Statistical analyses were performed using SAS ver. 9.2 (SAS Institute Inc. 2004). Standard analysis of variance (ANOVA) was performed using PROC GLM to determine variation in AUDPC_VS, AUDPC_RWCS and AUDPC_RWCL, RWCS_F and RWCL_F. The trait named RWCS_F represents the value of RWCS 15 days after watering withdrawal (which was the total extension of the drought period), whereas the trait RWCL_F refers to the value of RWCL for each RIL when the respective RWCS reached less than 23%. Variance components were estimated using PROC VARCOMP.

Broad sense heritability (h^2) that represents the part of genetic variance in the total phenotypic variance was calculated using the formula: $h^2 = \sigma_g^2 / (\sigma_g^2 + \sigma^2/r)$, where σ_g^2 is the genotypic variance, σ^2 is the error variance and r is the number of replications. Normality of residual distribution was checked using the Kolmogorov-Smirnov test. Pearson correlation coefficient between traits was estimated using PROC CORR in SAS.

Map Construction

A previous genetic map developed using the RIL population of the cross P665 x cv. Messire (Carrillo et al. 2014) was used for QTL analysis. This map covered 1119.46 cM and contained 414 markers: three morphological traits, one isozyme, 222 RAPDs (Random Amplified Polymorphic DNAs), 59 SSRs (Simple Sequence Repeats), 12 ESTs (Expressed sequence tags), 117 SNPs (Single nucleotide polymorphisms) and 21 SSRs (Simple sequence repeats) distributed in seven linkage groups (LGs).

The linkage map was constructed by MAPMAKER Version 3.0b (Lander et al. 1987) using a LOD score of 5.0 as the threshold for significant linkage. The marker orders were established using MSTMap (Wu et al. 2008) by finding the minimum spanning tree of a graph for each linkage group. MAPMAKER was used to confirm marker order determined by MSTMap. Recombination fractions were converted to centiMorgans (cM) using the mapping function of Kosambi (1944).

QTL analysis

QTL analysis was conducted using composite interval mapping (CIM) and multiple interval mapping (MIM) in Windows QTL Cartographer V2.5 (Wang et al. 2011). Markers to be used as cofactors for CIM were selected by forward-backward stepwise regression. The number of markers

controlling the genetic background in CIM was set to five. The thresholds for the detection of QTLs were estimated by permutations analysis (Churchill et al. 1994) using 1,000 permutations. One- and two-LOD support intervals for the position of each QTL were calculated as described by Darvasi et al. (1997).

To obtain more precise information on QTL effects and positions and to evaluate for the presence of digenic epistatic interactions across the QTL pairwise combinations, multiple-interval mapping (MIM) (Kao et al. 1999; Zeng et al. 1999), as implemented in WinQTL Cartographer, was used by considering as initial QTL models the CIM results obtained for the trait. The initial CIM-derived QTL model was subjected to a search for significant epistatic interactions among QTLs. Both main additive effects and their epistatic interactions were tested for significance using the Bayesian information criterion (BIC) with the penalty function $c(n) = \log(n)$, with n (sample size) = 111 (Zeng et al. 1999). The final main additive and epistatic QTL effects and the R^2 values of the model were then estimated.

RESULTS

Assessment of drought related parameters

The parent P665 showed very low drought symptoms with a mean AUDPC_VS value of 16.75, contrasting with the 24.13 of cv. Messire ($p < 0.05$) (Fig. 1a). The ANOVA revealed that the variation in AUDPC_VS among the RIL families was significant ($p < 0.001$), and a high value for broad sense heritability (h^2) was observed for this trait ($h^2 = 0.702$) (Table 1).

Table 1. Heritability of the different traits scored in the RIL population.

Trait	h^2^a
AUDPC_VS	0.702
AUDPC_RWCS	0.847
RWCS _F	0.856
AUDPC_RWCL	0.944
LRWC _F	0.697

^a h^2 : broad sense heritability.

Furthermore, transgressive RIL lines with increased susceptibility were identified, as it is shown by the frequency distribution (Fig.1a).

Fig. 1 Frequency distributions for the 5 traits AUDPC_VS (a), AUDPC_RWCS (b), RWCS_F (c) AUDPC_RWCL (d), and RWCL_F (e) in P665 x cv. Messire RIL population. Values for both parents are indicated by arrows.

AUDPC_RWCS and AUDPC_RWCL represented the variation on RWC in soil and leaves along the time course whereas RWCS_F and RWCL_F represented the values for these traits on the last time point sampled, corresponding to terminal water stress.

Parental lines, showed an average AUDPC_RWCS value of 965.8 in the case of P665 and 830.8 of cv. Messire (Fig. 1b). The average RWCS_F on the last time point observed for both parentals

was 32.9% for P665 and 20.0% for cv. Messire (Fig. 1c). However, no significant differences were found between P665 and cv. Messire for AUDPC_RWCS ($p = 0.519$) or RWCS_F ($p = 0.051$).

On the other side, the average AUDPC_RWCL for P665, characterized as drought tolerant on previous experiments (data not shown), was higher ($p < 0.05$) than the one observed for cv. Messire (267.0 vs 276.2, Fig. 1d). At the end of the water stress period cv. Messire, which showed a moderate susceptibility to drought, displayed an average RWCL_F value of 81.7% whereas for P665 was of 90.0% (Fig. 1e).

Variation in AUDPC_RWCS, AUDPC_RWCL, RWCS_F and RWCL_F among the RIL families was found to be highly significant according to ANOVA ($p < 0.001$). Transgressive RIL lines with increased tolerance and susceptibility were identified for RWCS_F, RWCL_F and AUDPC_RWCS. In addition, no transgressive lines with increased susceptibility were observed for the traits AUDPC_VS and AUDPC_RWCL (Fig. 1). Broad sense heritabilities (h^2) were high in all cases (Table1).

The correlation observed between traits, was found to be high for all traits except AUDPC_RWCL and AUDPC_VS ($r = 0.391$) but significant for all of them ($p < 0.001$) (Table 2).

Table 2. Pearson's correlation coefficients among the traits assessed.

Trait	AUDPC_VS	AUDPC_RWCS	RWCS _F	AUDPC_RWCL
AUDPC_RWCS	-0.620***			
RWCS _F	-0.598***	0.938***		
AUDPC_RWCL	-0.391***	0.614***	0.457***	
RWCL _F	-0.580***	0.780***	0.665***	0.803***

***, significance at the 0.1% nominal level.

QTL analysis

Quantitative trait loci analysis revealed a total of ten QTLs associated with drought tolerance traits. Two QTLs were associated with the variable AUDPC_VS (*audpc_vs-1*, *audpc_vs-2*), four with RWCS_F (*audpc_rwcs-1*, *audpc_rwcs-2*, *rwcs_F-1* and *rwcs_F-2*), and four with RWCL_F (*audpc_rwcl*, *rwcl_F-1*, *rwcl_F-2* and *rwcl_F-3*) (Fig. 2). Genomic positions, LOD scores and additive effects of each QTL are shown in Table 3.

Table 3. QTLs in the RIL population derived from cross P665 by cv. Messire.

Trait ^a	Linkage group	QTL	Flanking markers	Peak ^b	LOD ^c	Add ^d	R ² ^e
AUDPC_VS	III	<i>audpc_vs-1</i>	OPAA17_947 / AD57	57.12	3.38	-0.69	10.94
	I	<i>audpc_vs-2</i>	OPM4_978 / OPM6_486	61.56	3.32	-0.62	8.86
Total							19.80
AUDPC_RWCS	III	<i>audpc_rwcs-1</i>	Psblox2 / AB141	212.80	11.88	-53.77	31.29
	III	<i>audpc_rwcs-2</i>	OPAI14_1353 / OPAI14_1273	40.96	5.30	37.27	13.77
Total							45.06
RWCS _F	III	<i>rwcs_F-1</i>	A6 / OPW5_387	221.04	12.24	-4.15	32.89
	III	<i>rwcs_F-2</i>	AA175 / OPAI14_1353	40.42	6.94	3.07	19.64
		<i>rwcs_F-1 x rwcs_F-2</i>				-1.84*	4.77
Total							57.30
AUDPC_RWCL	III	<i>audpc_rwcl</i>	AB141 / AB64	215.21	7.36	-5.56	25.56
Total							25.56
RWCL _F	III	<i>rwcl_F-1</i>	OPW5_387 / OPAE5_538	223.59	8.01	-6.96	24.02
	III	<i>rwcl_F-2</i>	OPAI14_1353 / OPAI14_1273	40.96	4.85	4.78	11.37
	VI	<i>rwcl_F-3</i>	OPJ12_440 / AC74	7.33	3.73	4.46	9.01
Total							44.40

^a Traits assessed: AUDPC_VS: area under progress curve calculated for the mean values of the visual AUDPC_VS along the drought time course AUDPC_RWCS: area under progress curve calculated for the mean values of RWCS along the 15 days time course; AUDPC_RWCL: area under progress curve calculated for the mean values of RWCL along the water stress time course; RWCS_F: soil relative water content 15 days after watering withdrawal; RWCL_F: mean value of the RWCL for a 22% of average RWCS.

^b Peak: QTL position (cM);

^c LOD: peak LOD score from CIM;

^d Add: the additive effect from CIM (AUDPC_RWCL) or MIM (AUDPC_RWCS, RWCS_F, AUDPC_VS, LRWC_F).

^e R² (%): proportion of phenotypic variance explained by the respective QTL (for AUDPC_RWCS, RWCS_F, AUDPC_VS and LRWC_F the partial R² of each QTL was estimated from MIM).

* The value refers to the epistatic effects. Epistatic interaction between QTLs for RWCS_F was found significant in multiple-interval mapping (MIM) and included in the model.

The QTLs explained individually from 9 to 33% of the phenotypic variation depending on the variable and altogether from 20 to 57% using multiple-interval mapping (MIM) approach alleles conferring higher values of $RWCS_F/L_F$ and $AUDPC_RWCS/L$, or lower values of $AUDPC_VS$, and thus tolerance to water stress, were originated from P665 in case of all the QTLs except *audpc_rwcs-1*, *rwcs_F-1*, *audpc_rwcl* and *rwcl_F-1* for which the alleles promoting tolerance came from cv. Messire.

Significant pairwise epistatic interactions among the QTLs for $RWCS_F$ was found in multiple-interval mapping (MIM) and incorporated to the model.

DISCUSSION

QTLs have been mapped for a wide range of agronomic traits in pea, including biotic and abiotic stresses. QTLs for incomplete and complete resistance have been detected for the most important diseases affecting pea (see Rubiales et al. 2009a as a review) and for tolerance to abiotic stress such as winter frost and frost damage (Lejeune-Hénaut et al. 2008; Dumont et al. 2009). However, to the best of our knowledge, no previous study has addressed the genetics of tolerance to drought in pea or reported QTLs for this trait. For the first time in this work, the genetics and heritability of drought tolerance in pea is analyzed and the main zones of the genome controlling water stress tolerance have been identified. Differences between parental genotypes P665 and cv. Messire regarding water stress tolerance made it possible to assess visual symptoms along a water stress time course and analyze segregation of $RWCS/RWCL$ and $AUDPC_VS$ within the RILs.

Polygenic nature of drought tolerance has been pointed out in different studies in other crops based on a number of parameters associated with this trait (Bartels et al. 2005; Cattiveli et al. 2008). The continuous distribution observed in our study within the RIL population for drought tolerance in pea as well as the identification of 10 putative QTLs explaining from low to moderate percentage of the phenotypic variation also suggests a polygenic control of drought tolerance in pea.

Fig. 2 Pea genetic linkage map constructed from a population formed by 111 F_{6:7} recombinant inbred lines (RILs) derived from the cross between the *P. sativum* subsp. *syriacum* accession P-665 and the *P. sativum* subsp. *sativum* cv. Messire. Bar positions indicate locations of quantitative trait loci: outer and inner interval corresponding to 1-LOD and 2-LOD support interval are indicated as a full box and a single line, respectively.

Fig. 2 (cont.).

RWCS has been reported to be useful for phenotyping under drought stress either by itself (Granier et al. 2006) or in combination with other measurements (Aguirrezabal et al. 2006). In wheat, RWCS has found to be related with water spectral indices in different genotypes (Gutierrez et al. 2010), although RWCS has never been used as a parameter to discriminate between tolerant or susceptible genotypes till now. Our study uses RWCS as a suitable trait for assessing quantitative drought tolerance in pea and reports the genomic regions controlling the character. RWCS_F had a significant and strong negative correlation with AUDPC_VS which reflects the fact that plants showed more symptoms when water availability in soil was lower.

RWCL has been previously reported as a valuable and appropriate index to assess drought tolerance in a number of crops (Hunt et al. 1987; Tripathy et al. 2000; Siddique et al. 2000; Gutierrez et al. 2010). In pea subjected to drought stress, sensitive genotypes have been found to be more affected by the decline in RWCL than tolerant ones (Upreti et al. 2000). Despite other parameters such as leaf water potential and osmotic adjustment that have been traditionally used to assess plant water status as well as water and solutes transport in the soil–plant–atmosphere continuum (Kramer 1988), only RWCL takes into account the possible effect of both leaf water potential and osmotic adjustment. Thus, RWCL gives us an idea of how water and solutes move through the plant. A high negative correlation was also found for RWCL_F and AUDPC_VS.

It has been reported that a decrease in RWCL can be a main factor resulting in reduced growth in response to osmotic stress in pea (Alexieva et al. 2001). Our results are in agreement with these, as RWCL was a suitable parameter to evaluate drought tolerance in our RIL population and allowed the identification of the genomic regions controlling the trait. In our study RWCS_F and RWCL_F were highly correlated and two out of the three QTLs controlling RWCL_F co-localized with QTLs for RWCS_F, suggesting a common genetic control which could be explained by their relation with the water transport along the plant.

Strong correlation between AUDPC_VS and RWCL_F in soil and leaves, respectively, points out the visual scale as a proper tool to assess different aspects and traits related to drought tolerance. Accordingly, correlation was higher when we were referring to the last point of the time course of both

RWCS and RWCL (RWCS_F and RWCL_F) instead of their respective AUDPC values, as the symptoms observed were more intense at the end of the drought period, when there is less water available in soil and plant tissues.

In the RIL population the distribution was skewed towards lower values of RWCS_F/L_F and AUDPC_RWCS/L, with many families showing lower values of these traits than cv. Messire. This fact suggested the existence of some QTLs that had alleles promoting high RWCL and RWCS under water stress also in the susceptible cv. Messire. These more susceptible lines in this RIL population would be those lacking favourable alleles at four of the QTLs associated with high relative water content (*audpc_rwcs-1*, *rwcs_F-1*, *rwcl_F-1* and *audpc_rwc*) derived from cv. Messire. In fact, we had observed in previous studies (data not shown) that cv. Messire preserved a moderately high relative water content under water stress when compared with genotypes highly affected by drought, indicating that cv. Messire was moderately susceptible. Our present results support the hypothesis that genes conferring high RWCS/L in cv. Messire and in P665 were different. Some transgressive RIL families with higher RWCS_F/L_F and AUDPC_RWCS values than both parents were identified. These RIL families which showed a higher RWC than P665 might possess favourable alleles for all tolerance QTLs. This would be in agreement with the negative epistatic effect observed in the case of QTLs *rwcs_F-1* and *rwcl_F-2* given the fact that the complementary favourable alleles were in repulsion phase.

Through our study, we were able to clearly distinguish the genomic regions controlling drought tolerance in each parental line. Thus, the QTLs promoting tolerance from cv. Messire were all located in the distal part of LGIII. By contrast, QTLs for drought tolerance from P665 (*audpc_rwcs-2*, *rwcs_F-2*, *rwcl_F-2* and *rwcl_F-3*) were located in three different regions, one at the beginning of LGIII and two in LGI and LGVI.

Although the QTLs identified explained a high proportion of phenotypic variation for most of the traits, they could not explain all the observed variation. Nevertheless, it is possible that the parents possess some common tolerance genes and that a QTL analysis in RILs from cross with an accession more susceptible than cv. Messire may allow detection of additional genes which might be governing high RWCS/L within the segregating population. In addition, the measurement of additional parameters and the use of other assessment tools for traits related with drought tolerance may allow

us to better unravel the complexity of the genetic networks implied on the physiological responses in the plant.

The population used in our study had been previously used to identify QTLs for other traits that could be related to drought resistance such as root length, blooming date or aerial plant biomass (Fondevilla et al. 2011). Three QTLs associated with RWCS/L (*rwcl_F-1*, *rwcl_F-3* and *rwcs_F-1*) were in the same genomic region as two QTLs controlling resistance to *O. crenata* in this same RIL population (Fondevilla et al. 2011). Thus, *rwcl_F-1* and *rwcs_F-1* were mapped exactly in the same region of LGIII as the QTLs *n°br03_2*, and *rwcl_F-3* was in the same region of LGVI as the QTLs *n°br04_1*. Both *n°br04_1* and *n°br03_2* are QTLs for *O. crenata* incomplete resistance under field conditions. Interestingly, the parasitic plant *O. crenata* obtains nutrients, but also water from its pea host. Therefore, water and solutes loss produced by drought and by *O. crenata* attack could be controlled by similar genomic regions or maybe these genomic regions also control phenology, root morphology and other traits that contribute to reduction of both drought and broomrape damages. Supporting these hypotheses, the alleles promoting higher RWC and resistance to *O. crenata* would come from cv. Messire in the QTLs *rwcl_F-1*, *rwcs_F-1* and *n°br03_2*, whereas the tolerance to drought and the resistance to *O. crenata* associated to the QTL *rwcl_F-3* and *n°br03_2*, respectively, would be conferred by P665.

Furthermore, the QTLs *rwcl_F-1* and *rwcs_F-1* were located in the confidence interval of the QTL *dfIII.1*, associated to earliness (Fondevilla et al. 2011), which would also allow avoiding seasonal drought stress (Forres et al. 2010). Although blooming date was obtained in field conditions, earliness could be meaningful as differences in growing patterns between lines could be due to a different drought tolerance/sensibility level. Also, knowing this could be helpful in the process of selecting material both for tolerance and escape to drought. Other QTLs such as *MpIII.1_DRseedI*, both associated with incomplete resistance to *D. pinodes* and *Psy1*, associated with *Pseudomonas syringae* pv. *syringae* resistance, are located in this region (Fondevilla et al 2011; 2012), which could mean that these genes are involved both in biotic and abiotic stress. In this case, the alleles promoting drought tolerance came from P665, whereas cv. Messire conferred the resistance to *D. pinodes* and the earliness.

Among the QTLs associated with RWC derived from P665, *rwcl_F-2* (LGIII), *rwcs_F-2* (LGIII) and *audpc_rwcs-2* (LGII), explained the 11.37, 19.64 and 13.77 % of the phenotypic variability for this trait, respectively. In addition, these QTLs were located in the same genomic region as a QTL (*rl3*) associated with higher root length in cv. Messire, but also with another QTL, *dfIII.2*, related to earliness in flowering tested under field conditions in a previous study (Fondevilla et al. 2011). Furthermore, this genomic region was found to be associated with the QTLs *MpIII.3_DRI_05*, *MpIII.3_DRst_05*, *MpIII.3_DRI_06* and *MpIII.3_DS_06* all of them related to *D. pinodes* resistance (Fondevilla et al. 2011). It seems that precocity and *D. pinodes* resistance are somehow related in field conditions, and it is possible that these QTLs are more related to flowering than associated to genetic resistance to *D. pinodes per se*. Both QTLs for earliness and *D. pinodes* resistance came from P665.

The relationship between RWCS/RWCL and roots has been previously observed in rice, where the genes controlling resistance to drought and root morphology traits were detected in the same region (Yue et al. 2005). In fact, root morphology is widely known as a trait related with drought avoidance (Grzesiak et al. 1997; O'Toole et al. 1987; Ludlow 1989). Selection for deep and extensive root system has been advocated to increase productivity of food legumes under moisture deficit conditions as it can optimize the capacity to acquire water. Turner et al. (2001) identified rooting depth and density as a main drought avoidance trait in grain legumes for use in terminal-drought environments. Therefore, it was highly significant that SRWC and root length QTLs were located in the same region in our study.

SSRs and SNPs are robust tools for marker assisted selection (MAS). In our study *rwcs_F-1* was associated to the SSR marker A6 whereas *rwcl_F-2*, *audpc_rwcs-2* and *rwcs_F-2* were in the vicinity of the SSR marker AA175. In addition, *rwcl_F-3* and *audpc_vs-1* were flanked on one side by AC74 and AD57, respectively, and *audpc_rwcl* was flanked by AB141/AB64, all of them SSR markers which can be used to detect these QTLs. SSR markers are locus-specific, easy to score due to the absence of similar sized interfering fragment, low sensitive to reaction conditions and reproducible. Therefore, the SSR markers associated with the drought tolerance QTLs could be useful for MAS in drought tolerance breeding programs.

Gene-based markers located in the vicinity of *audpc_vs-1*, *audpc_rwcs-1* and *rwcs_F-1* were also identified in our study. In the case of *audpc_vs-1* it was flanked by the SNPs PsAAP2 and DipeptIV which correspond to the proteins *Aminoacid permease II* and *Dipeptidyl peptidase IV like-protein*, related with general metabolic process such as aminoacid transport and proteolysis. On the other hand, *audpc_rwcs-1* was flanked by *Psblox2*, which is an SSR corresponding to a lipoxygenase, enzyme whose genetic expression is related to wounds, defense or water deficit response (Bell and Mullet 1991; Aubert et al. 2006) and has interestingly found to be associated to drought stress tolerant peanut genotypes (Kottapalli et al. 2009). *Psblox2* could thus be considered a proper candidate gene for drought tolerance in pea.

In addition, the presence in our map of molecular markers common to other pea genetic maps made it possible to compare the QTLs identified in our study with those reported in different populations. Therefore, we found that AA175 had been associated with the Meta QTL *Ae8-9(Ae-Ps3.1)* for *Aphanomyces euteiches* resistance (Hamon et al. 2013), a consensus genomic region which would allow the selection for several agronomically interesting traits. Interestingly, Hamon et al. (2013) related this region with *Fwn3.1*, a QTL associated with resistance to *Fusarium oxysporum* f. sp. *pisi* race 2 (McPhee et al., 2012) also a fungal soil pathogen. Both *A. euteiches* and *F. oxysporum* produce vascular wilting, which triggers similar symptoms as water stress at the plant level.

Furthermore, in the distal part of the chromosome III, *audpc_rwcl* was flanked by the SSR marker AB64, which is also comprised in the LOD2 region of the QTL *rwcs_F-1* and matches with a hot spot pointed out by Prioul-Gervais et al. (2007). In this region would be included *Fwn3.2*, a QTL associated with resistance to *F. oxysporum* f. sp. *pisi* race 2 (McPhee et al. 2012) and the *Fw* gene, conferring resistance to *F. oxysporum* f. sp. *pisi* race 1 (Dirlewanger et al. 1994; Weeden et al. 1998), which produces the same symptoms as race 2. The significant epistatic interaction observed between *rwcs_F-1* and *rwcs_F-2* can be meaningful from this perspective, as a synergistic effect between these two regions could lead to a higher protection against the desiccation promoted by the fungus.

Finally, both *audpc_rwcl* and *rwcs_F-1* would be also in the LOD2 region of the “*Le*” gene (Lester et al., 1997; Bordat et al. 2011), which presence in this zone would be quite significant regarding drought tolerance. This gene encodes a Gibberelin 3P-hydroxylase, an enzyme related with activation

of the plant hormones gibberellins (GAs) and traditionally associated with growth regulation (Lange et al. 1999), but also found to be implied in stress protection (Vettakkorumakankav et al. 1999) and modulation (Alonso-Ramírez et al. 2009). Hormones such as ethylene, salicylate, jasmonate and abscisic acid (ABA) act synergistically or antagonistically to regulate plant responses to pathogens and abiotic stress factors (Rao et al., 2000, 2002; Borsani et al. 2001; Turner et al. 2002; Xiong et al. 2002). Thus, the presence of QTLs related to drought stress in this region would be in agreement with the QTLs described in previous studies and also support the biotic and abiotic stress connections on the gene level. Furthermore, “*Le*” could be a good candidate gene for drought tolerance. The development of single nucleotide polymorphism (SNP) markers as well as single-stranded conformation polymorphism (SSCP) markers for genes encoding enzymes involved in primary metabolism made it possible sometimes to compare genetic maps between pea and the model legume *Medicago truncatula* (Aubert et al. 2006; Bordat et al. 2011; Duarte et al. 2014). For example, in our study we observed that *AUDPC_VS-2* was located in the region between *PsAS2_SNP1* and *Rgp_SNP2*, whereas according to Aubert et al. (2006) in the correspondent region of the chromosome V of *M. truncatula* (between *PsAS2* and *Rgp*) will be *ADWmant.5*, a QTL associated to the SSR marker MTIC238 and related to aerial dry weight under osmotic stress (Badri et al. 2011). However, further saturation of both maps, including markers in the LOD2 region of both QTLs would be necessary to confirm any relationship.

The present study identified ten QTLs from pea which are related to different aspects of drought stress. The knowledge of the genetic system controlling tolerance to drought in accession P665 and cv. Messire and molecular markers linked to the genes controlling the trait would facilitate drought tolerance gene transfer into elite pea cultivars in MAS schemes.

AUTHOR CONTRIBUTION STATEMENT

Iglesias-García R: Study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript.

Prats E: Study conception and design, analysis and interpretation of data, drafting of manuscript.

Fondevilla S: interpretation of data and drafting of manuscript.

Satovic Z: Analysis of data, critical review.

Rubiales D: Study conception, drafting of manuscript, critical review.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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