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M. AFIFUDDIN LATIF ADIREDJO

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Directeur(s) de Thèse :

M. PHILIPPE GRIEU

M. THIERRY LAMAZE

Rapporteurs :

M. JEAN-MARC GUEHL, INRA NANCY

M. THIERRY SIMMONNEAU, MONTPELLIER SUPAGRO

Membre(s) du jury :

M. SYLVAIN CHAILLOU, AGROPARISTECH, Président

M. NICOLAS LANGLADE, INRA TOULOUSE, Membre

M. PHILIPPE GRIEU, INP TOULOUSE, Membre

M. THIERRY LAMAZE, UNIVERSITE TOULOUSE 3, Membre

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SOMMAIRE

L'efficience d'utilisation de l'eau (WUE), rapport entre la biomasse produite et l'eau consommée, est un trait essentiel à étudier en agronomie pour améliorer la production des cultures soumises à la sécheresse. Cependant, mesurer l'eau consommée par un couvert végétal est difficile à réaliser. L'objectif général de ce travail de thèse est de répondre à trois principales questions : (i) peut-on déterminer WUE en utilisant la discrimination isotopique du carbone (CID) facile à mesurer? (ii) comment l'analyse de la variabilité de WUE et CID peut contribuer à sélectionner des génotypes de tournesol soumis à la sécheresse? (iii) et les variations de WUE peuvent-elles être révélées par les variations de relations hydriques?

Quatre expériences ont été conduites en serre pendant deux années : (i) avec deux scenario de sécheresse, l'une progressive, l'autre stable, et (ii) avec cinq niveaux de contenu en eau du sol stables. Les principaux traits mesurés sont WUE, CID et d'autres traits représentant les relations hydriques tels que le contrôle de la transpiration (FTSWt), la capacité d'extraction de l'eau (TTSW) et la tolérance à la déshydratation (OA).

Une forte corrélation négative a été mise en évidence entre WUE et CID. Une large variabilité a également été observée pour FTSWt, TTSW et OA. Ces résultats permettent de connaitre le contrôle génétique de WUE et CID, ainsi que des traits associés, lesquels n'ont jamais été relatés dans la littérature. De plus, l'analyse QTL pour FTSWt n'a pas non plus été réalisée, chez aucune plante.

Des QTL pour WUE et CID ont été identifiés pour différents scenario de sécheresse. Les QTL pour CID sont considérés comme 'constitutifs', parce qu'ils sont détectés dans les différents scenarios. Les QTL pour CID co-localisent avec ceux pour WUE, pour la biomasse et pour la transpiration cumulée. Des co-localisations de QTL ont également été observées entre FTSWt et TTSW, entre TTSW et WUE-CID-Biomasse, et entre FTSWt-TTSW et Biomasse.

Cette étude met en évidence que WUE est physiologiquement et génétiquement associée à CID. De plus, CID représente un excellent substitue à la mesure de WUE et permet d'améliorer l'efficience d'utilisation de l'eau par sélection assistée par marqueurs.

SUMMARY

Water use efficiency (WUE), measured as the ratio of plant biomass to water consumption, is an essential agronomical trait for enhancing crop production under drought. Measuring water consumption is logistically difficult, especially in field conditions. The general objective of the present Thesis is to respond to three main questions: (i) can WUE be determined by using carbon isotope discrimination (CID), easy to measure?, (ii) how WUE and CID variation analysis can contribute to the genotypic selection of sunflower subjected to drought?, and (iii) can WUE variation be revealed by the variation of plant-water relation traits.

Four experiments were carried out in greenhouse across two different years: (i) on two drought scenarios, progressive soil drying and stable water-stress, and (ii) on five levels of soil water content. The main traits that have been measured include WUE, CID, as well as plant-water relation traits, i.e. control of transpiration (FTSWt), water extraction capacity (TTSW), and dehydration tolerance (OA).

A highly significant negative correlation was observed between WUE and CID, and a wide phenotypic variability was observed for both WUE and CID. A wide variability was also observed for FTSWt, TTSW and OA. The results provide new insight into the genetic control of WUE and CID related-traits, which, unlike to other crops, genetic control of WUE, CID, and TTSW in sunflower have never been reported in the literature. Further, quantitative trait loci (QTL) mapping for FTSWt was never reported in any plant species.

The QTL for WUE and CID were identified across different drought scenarios. The QTL for CID is considered as a ‘‘constitutive’’ QTL, because it is consistently detected across different drought scenarios. The QTL for CID co-localized with the QTL for WUE, biomass and cumulative water transpired. Co-localization was also observed between the QTL for FTSWt and TTSW, between the QTL for TTSW and WUE-CID-biomass, as well as between the QTL for FTSWt-TTSW and biomass.

This study highlights that WUE is physiologically and genetically associated with CID. CID is an excellent surrogate for WUE measurement, and can be used to improve WUE by using marker-assisted selection (MAS) to achieve the ultimate goal of plant breeding at genomic level.

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1 INTRODUCTION

Sunflower (*Helianthus annuus* L.) has been widely regarded as a plant able to grow under low water-input regimes (Merrien et al. 1981; Connor and Jones, 1985; Unger, 1990; Chimenti et al. 2002). This ability is mainly due to deep roots able to extract or use water efficiently from the deeper parts of the soil (Connor and Hall, 1997; Mwale et al. 2003; Karam et al. 2007; Roche et al. 2009). The ability of sunflower genotypes to use water is not homogeneous and is known to be variable (Merrien et al. 1981). Nevertheless, genotypic variability of sunflower water use has rarely been explored, especially in drought condition (Poormohammad Kiani, 2007a; Casadebaig et al. 2008). Therefore, there is pressing need to understand the variability of sunflower water use for achieving the goal of genotypic selection and crop improvement program of sunflower subjected to drought.

Drought induces a range of physiological and morphological responses in plants. The main responses include: reducing water loss through stomatal control of transpiration (Yeo, 2007), accumulating of compatible solutes (Flowers, 2007), maintaining water uptake through an extensive root system (Kavar et al., 2007), protecting the roots from excessive water loss by decreasing root hydraulic conductivity (Steudle, 1994, 2000), and limiting the number and area of leaves (Schuppler et al. 1998). Intensive studies have been carried out to identify plant responses to drought for obtaining criteria for the selection of drought-tolerant plant (Price et al. 2002; Clavel et al. 2005; Martínez et al. 2007; Karaba et al. 2007; Hessini et al. 2009). Many of these criteria are not only based on plant-water relations, e.g. cumulative water transpired on a period of growth, control of transpiration, water extraction capacity, hydraulic conductivity, and dehydration tolerance, but also based on production, e.g. photosynthesis, biomass, and leaf area (Losch, 1993; Blum et al.1996; Sánchez et al. 1998; Lizana et al. 2006).

The relationship between water relations and production has generated interest for agronomist, physiologists and breeders to study the water use for crop plants (Sinclair et al. 1984; Ehleringer et al. 1993; Condon et al. 2004). In the last years, considerable efforts were made to study the physiology and the genetic control of water-use efficiency (WUE) and much attention has been devoted to the understanding of physiological processes that control this character (Condon et al. 2004; Hessini et al. 2009). WUE is traditionally defined either as the ratio of biomass accumulation to water use over a season, or as the ratio of photosynthesis

(*A*) to transpiration (*E*) over a period of seconds or minutes (Condon, 2004). This trait can contribute substantially to crop productivity when water is limited (Wright et al. 1994). It is suggested that a way of enhancing plant performance in drought conditions is to improve the WUE (Condon et al. 2004). However, WUE is difficult (i.e. the lack of simple method) and time consuming to measure, especially in field condition because water use is difficult to be determined in field conditions. The difficulty of measuring WUE has been overcome since Farquhar and colleagues (Farquhar et al. 1982; Farquhar and Richards, 1984) proved the negative relationship between WUE and carbon isotope discrimination (CID) in tissue of C_3 plant species. CID has been demonstrated to be a simple but reliable measure of WUE, and negative correlation between them has been used to indirectly estimate WUE under selected environments (Ehleringer et al. 1993). In addition, CID is defined as a measure of the ratio of the stable isotopes of carbon ($^{13}C/^{12}C$) in plant material relative to the value of the same ratio in the atmosphere (Farquhar et al. 1989, Condon, 2004).

The genetic selection for specific physiological and morphological traits in drought environments, such as a high WUE and a low CID is seen as providing part of the solution (Fussel et al. 1991; Richards, 1996; Richards et al. 2002). Genetic selection of WUE and CID could be enhanced with a better understanding of its genetic control (Chen et al. 2011). The advancement of computational methods and DNA-based molecular markers in the late 1980s and 1990s has revolutionized the dissection of quantitative trait inheritance (Baum et al. 2007). Quantitative trait, such as WUE and CID, is governed by quantitative trait loci (QTL) (Hall et al. 1994; Mian et al. 1996; Juenger et al. 2005). The QTL analysis provides opportunities to identify and locate chromosome regions controlling WUE and CID during plant growth in water-limited conditions (Mian et al. 1998). The ultimate goal is to manipulate beneficial QTL alleles through marker-assisted selection (MAS) to derive improved sunflower genotypes and accelerate plant breeding under drought environments.

In the present Thesis, the general objectives are to respond to three questions: (i) Can WUE be determined by using carbon isotope discrimination (CID)?, (ii) How WUE and CID variation analysis can contribute to the genotypic selection of sunflower subjected to drought?, and (iii) can WUE variation be revealed by the variation of plant-water relation traits, i.e. control of transpiration, water extraction capacity, dehydration tolerance and root hydraulic conductance?. The general objectives are divided in four specific objectives that represent the objectives of four publications derived from the present Thesis: (i) to identify the genetic

control of WUE and CID by using QTL mapping in a population of RILs of sunflower, and to compare QTL associated with these traits in a dual drought scenario, a progressively water-stressed establishment, and a stable water deficit treatment, (ii) to investigate the physiological behaviors and to analyze the genetic control of plant-water relation traits, i.e. transpiration control, water extraction capacity and dehydration tolerance, (iii) to evaluate root hydraulic properties in four sunflower RILs differing in WUE, and (iv) to explore the possibility of using CID as an indicator to select sunflower genotypes with high WUE, by assessing the relationship between CID and WUE in four RILs of juvenile sunflowers, and by evaluating the CID and WUE at five levels of SWC which were maintained stable during the experiments.

2 LITERATURE REVIEW

2.1 Sunflower

2.1.1 Historical aspects and the utilization of sunflower

The domesticated sunflower was introduced from North America into Europe by the early Spanish explorers in 1510, where they initially gained popularity as a garden ornamental. The agronomic development of sunflower as an oilseed crop and for use as edible achenes (confectionery types) took place in Russia, where a number of landraces had been developed by the late 1800s. Initial selection emphasis was given to early maturity, disease and pest resistance, and high seed oil content. Sunflower was reintroduced from Russia to North America in the later part of the 19th century (Putt, 1997).

The common sunflower (*H. annuus*) is the most important species grown commercially, although other species are also cultivated, e.g. *H. tuberosus*, which is grown for production of edible tubers, and several other species grown as ornamentals. The name *Helianthus* is derived from the Greek words ‘‘helios,’’meaning sun, and ‘‘anthus,’’ meaning flower. The Spanish name for sunflower, ‘‘girasol,’’ and the French name ‘‘tournesol’’ literally mean ‘‘turn with the sun,’’ a trait exhibited by sunflower until anthesis, after which the capitula (heads) face east (Fernandez-Martinez et al. 2009). Wild *H. annuus* was used for food by the Native American Indians and, due to its association with humans, it became a camp-following weed that was introduced into the central part of the U.S., where it was domesticated and carried to the east and southwest (Heiser et al. 1969; Heiser, 1978).

Nowadays, sunflower (*Helianthus annuus* L.) is one of the most widely cultivated oil crops in the world (Flagella et al. 2002; List, 2014). There are two types of sunflowers: oil types containing about 40% oil and non-oil types with about 30% oil. Oil types represent 80–95% of sunflower seed production. The oil is mainly used for cooking and frying. Industrial uses include lighting, paints, cosmetics, resins, lubricants and biofuel. Non-oil types are mostly used in confectionery products such as roasted seeds (FAO, 2010; Ghobadi et al. 2013).

2.1.2 Sunflower production in France and in the world

France is the first sunflower producer in the European Union with 1.64 million tons of seeds in 2010. About 80% of the French sunflower seeds production goes for domestic uses

(Labalette and Raoul, 2012). Despite sunflower hectares (ha) have largely decreased during the last 20 years because of an insufficient profitability, real increases in planting have been observed since 2008 due to the Common Agriculture Policy evolution and of higher selling prices for oil crops (Jouffret et al. 2012). From 2007, sunflower cultivation increased by 35 % in France to reach 695000 ha in 2010 (735000 ha in 2011) mainly due to good prices and to a more favourable European agricultural policy (Labalette and Raoul, 2012).

According to research of CETIOM[1], sunflower cultivation has a benefit over the last 25 years, is a real genetic progress. Through a series of tests divided into the main areas of production, it has increased 0.5 q/ha per year, or 1.3% annually (Fig. 2.1). However, the gap between the potential yield and the yield in culture remains important due to some limiting factors such as water supply, date of sowing, and fungi of *Phoma* (Anonymous, 2013).

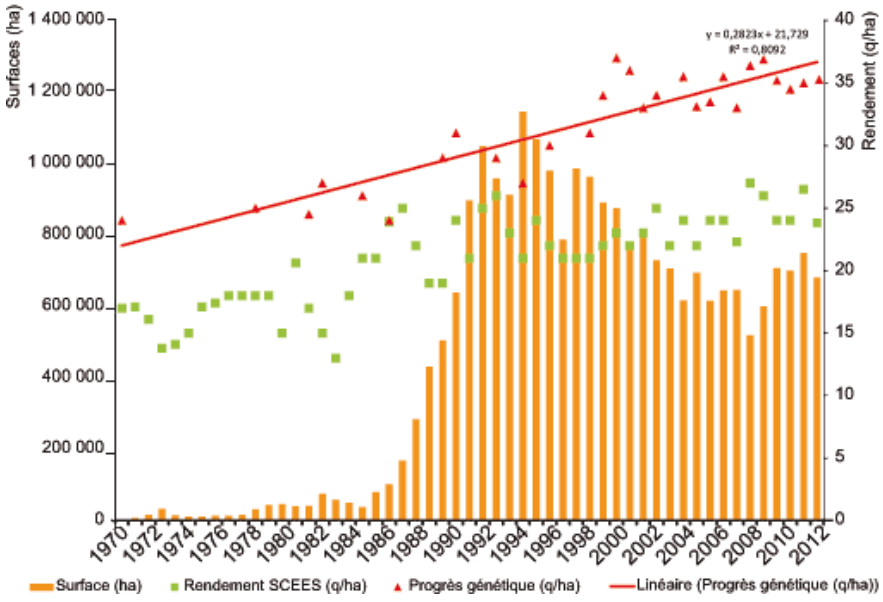

Figure 2.1 Margin between genetic potential and performance of culture (CETIOM, 2013).

Labalette et al. (2012) reported that the important sunflower areas in the world are Ukraine, Russia, EU-27[2], Argentina and Turkey. They also reported that particularly for oleic sunflower from these countries have quite doubled in five years, from around 500000 to 950000 ha. Further, the oleic types are mostly introduced in the South-Western Europe and in Hungary where their proportion reach around 30 % of the total sunflower acreage meanwhile the eastern countries starts only cultivating such types (1% of the total surfaces).

[1] Centre technique interprofessionnel des oléagineux et du chanvre
[2] The European Union of 27 member states

2.2 Drought

2.2.1 Definition and characterization

For agronomist, a drought is defined as any lack of water which the crops could not able to express their performance and yield in a favorable conditions, or which may affect the quality of the harvested products (Ludlow and Muchow, 1990). A tolerant plant in this case is the plant that is able to produce an output (plant production or yield) as high as possible in a given drought scenario (Zhu, 2002; Alqudah et al. 2011). Drought is characterized by its intensity, dynamics (suddenly or gradually implemented), duration and time of occurrence relative to the crop cycle. These preliminary remarks have a consequence: it is very difficult experimentally to identify specific characteristics of drought which agronomist faces (Chaves et al. 2003).

Drought occurs by the combination of (i) the restriction of the availability of soil water and (ii) the increase of the evaporative demand in the air. Water crosses the plant (transpiration) due to a difference in water potential between the air and the soil (Yeo, 2007; Barnabas et al. 2008). Drought intensity at a given time can be physically characterized by water status in the atmosphere and in the soil (Sinclair and Ludlow, 1986). The water status of the air is easy to be determined. The water potential in air is linked to the degree of saturation of the air with water vapor. Another usual physical parameter is vapor pressure deficit (VPD). However, plant transpiration depends on other environmental parameters such as the wind speed. In the soil, there is a high heterogeneity of water status, and its measurement is difficult to be implemented, especially in the field (Jones, 2013). The water status of the plant is related to the difference between the flow of water that enters through the roots and which flows out of the leaves in the same time (Comstock, 2002). Thus, drought experienced by the plant is defined, at every moment, by water conditions at the terminals of the plant, soil and air (Blum, 1998).

2.2.2 Plant responses to drought

Genotypes subjected to the same water deficit do not perceive the stress in the same way. A wide range of mechanisms has been summarized by Tardieu et al. (2007). In addition, a significant genotypic variability is associated with these mechanisms in many crops, especially in sunflower species.

Short-term response

At the scale of a few minutes, the plant can reduce transpiration by closing its stomata that reduces stomatal conductance (g_s) which determines gas exchange (CO_2 and H_2O) (Lauteri et al. 1997). For sunflower, this regulation seems different according to the growth stage, before or after anthesis (Connor and Hall, 1997). Before anthesis, sunflower is known to have a stomatal closure less sensitive to drought than most other species. Therefore, plants may lose water and reduce turgor, and is called "anisohydric" (see Section 2.2.4). In contrast, after anthesis, stomatal closure is more sensitive to water stress and appears as a key process to control plant water status. The stomatal closure is often accompanied by a reduction in CO_2 assimilation in the leaves (by decreasing the diffusion) and it increases the temperature of the leaves (the transpiration flow contributes to the dissipation of radiation energy) (Gollan et al. 1985; McDonald and Davies, 1996). However, in sunflower, maintaining high photosynthesis is a major process for obtaining a high yield. Poormohamad Kiani et al. (2007a) reported that there was high genotypic variability for g_s and net CO_2 assimilation (A) in a sunflower population grown under field condition at Toulouse. Moreover, different thresholds for stomatal closure have also been reported by Casadebaig et al. (2008) as a function of the fraction of transpirable soil water (FTSW) that represents a normalized way to express soil water content (SWC) (see chapter 3; Materials and Methods). There was a genetic difference in genotypes performance in term of control of transpiration rate (transpiration per unit leaf area).

Regulation of g_s is the major mechanism involved in the short term to limit water loss: early stomatal closure, maintains high leaf water potential (Medrano et al. 2002). Reduction in cellular leaf elongation is one of the earliest processes affected by water stress but the instantaneous effect on water lost is weak. Different genotypes may show different leaf water potentials (Mojayad, 1993) and growth stages (Morizet and Merrien, 1990) under similar drought conditions. Regulation of g_s also depends on: (i) a given instant, and (ii) the leaf water potential and air humidity in the field. Genotypes with low g_s are more sensitive to the vapor deficit in the air and to the lower leaf water potential than genotypes with high g_s. Low g_s is generally proposed as a favorable trait to adaptation to drought (Jones and Rawson, 1979; Jones and Corlett, 1992). If the stomata are not totally closed, due to the difference between the diffusion coefficients of water and CO_2 in the leaf, transpiration will decrease more than A: water use efficiency (WUE) will increase (Singh and Reddy, 2011).

In sunflower, decrease of intercellular CO_2 concentration may be accompanied by a sustained reduction in the chloroplast efficiency to use CO_2, even if subsequently the CO_2 availability is restored (D'Andria, 1995; Grieu et al. 2008). This functional alteration of chloroplast, more or less rapidly reversible, can contribute to reduce the daily balance of *A* of some genotypes, following the depression of g_s in mid-day or in post-drought (Wise et al. 1991).

Stomatal conductance is also closely related to the hydraulic conductivity of plant tissue, including roots (Lp_r). Stomata control involves chemical type messages that pass from the roots to the leaves by xylem sap, especially H+ (pH of the sap) (Wilkinson and Davies, 1997) and abscisic acid (ABA) synthesized by the non-dry organs (Davies and Gowing, 1991; Tardieu and Davies, 1993). Transformed plants which synthesize more of this hormone maintain a more favorable water status and survive longer in water deficit (Morgan, 1990). An important consequence of this mechanism is that the plant reduces its transpiration prior to leaf cellular dehydration (Borel et al. 2001). In sunflower, the Lp_r is generally considered to be high as compared to other field crops. Some researches in sunflower showed the important role of aquaporins, (AQPs: trans-membrane proteins) on the root Lp_r (Ouvrard et al. 1996). Trans-membrane proteins, AQPs, can modulate the path of water through cell membranes (Maurel et al. 2002; Luu and Maurel, 2005). Control of the AQPs is still imperfectly known, but it is clear that the water deficit and ABA affect the root Lp_r (Socias et al. 1997; Zhu et al. 2005). Additionally, higher Lp_r is associated to lower difference between the soil and leaves water potential (Carvajal et al. 1999, Steudle, 200; Christmann et al. 2007).

Middle-term responses (hours, days)

At the scale of a few hours to a few days, the plant can maintain tissue turgor through various mechanisms. The growth process is particularly sensitive to dehydration and performance of plants subjected to water stress. It depends not only on the ability of the acquisition of water, but also on the ability of organs to maintain their physiological functions (Morgan, 1984). Maintaining turgor during water deficit contributes to limit the negative effects of water stress on g_s and photosynthesis (Maury et al. 2000) as well as cell expansion (Cosgrove, 1986) and growth (Barlow, 1986), in particular the roots (Passioura, 1983). In sunflower, osmotic adjustment (OA), the ability to actively accumulate solutes in cell vacuoles, contributes to

drought tolerance, mainly by allowing extraction of more water from the soil and retaining this water in the tissues (Chimenti et al. 2002).

Leaf development decreases in water deficit condition before any reduction in photosynthesis (Boyer, 1970; Sperry et al. 2001). As turgor is the driving force of growth, many genetic programs aimed at improving their maintenance at water deficit via the accumulation of solutes in the cell. Other mechanisms are involved in maintaining the growth such as cell division (Granier et al. 2000), the mechanical characteristics of cell walls (Cosgrove, 2005), maintaining a high Lp_r through membrane proteins, AQPs (Tyerman et al. 2002; Maurel et al. 2008) and hormone signals (Sharp, 2002).

Long-term responses (weeks)

At the scale of a few weeks, the plant acclimates through morphological modification in addition to physiological adaptation and adjusts its transpiration by decreasing leaf area which will cause the reduction of plant production (Lambers et al. 2008). This is an adaptive mechanism which is to limit the development of leaves tissues. Similarly, a major consequence for the roots: is the maintenance of growth that allows exploring deeper soil layers where water supplies are essential (Kramer and Boyer, 1995; Hund et al. 2009).

During early drought, the decrease of leaf area is closely associated with the decrease of leaf expansion; the association is closer rather than with acceleration of leaf senescence, but, the acceleration of leaf senescence will also contribute to lower leaf area. In sunflower, this decrease may be followed by the decrease in performance if the leaf area index falls below 2.5 at flowering (Merrien and Grandin, 1990). Performance is correlated to the leaf area development after flowering, and is strongly affected when senescence is accelerated by late water deficits (Poormohammad Kiani, 2009).

The optimization of the water absorption is related to the complex of morphological characteristics of roots: mass, volume, and branching depth (Ramanjulu and Bartels, 2008). Many plants that adapt to arid area do not control their water loss through transpiration, but have very deep roots which can extract water from the soil. Root growth in dry conditions can be maintained by OA which limits the drop turgor in potential (Turner, 1986; Schachtman and Goodger, 2008). However, two types of reasons limit the use of many root criteria by breeders (Turner et al. 2001): (i) the impracticality of field screening for this feature on a large scale

and the difficulty of correlating field observations to those made in pots, (ii) the lack of a precise understanding of the exact role of the roots in water limited conditions is another limitation factor at the establishment of a screening system (Passioura, 1994).

2.2.3 Strategy of plant in response to drought

Several mechanisms expressed more or less effectively and occur simultaneously in plant during drought adaptation. According to the combination of mechanisms of plant response to drought, different behaviors (i.e. strategies) may be defined (Jones, 2013).

A first major way to avoid drought is "drought escape" or "drought avoidance". Drought escape allows the plant to avoid water stress by a well adaptation of the plant cycle to length of the rainy season (Jones, 2013). The rapid development with early flowering allows the plant to avoid dry periods. This strategy was applied to crops by moving sowing date and/or select early sowing varieties to avoid water deficit at the end of growth cycle (Farooq et al. 2009). A second major and general way to adapt is the ability of plants to maintain high water status by preventing dehydration. The strategy is mainly based on: (i) reduction of transpiration (Schuppler et al. 1998; Connor, 2005), (ii) an optimization of the water absorption through the roots (Turner et al. 2001), and (iii) the ability to maintain water in cells through OA (Zhang et al. 1999). Among these strategies, reduction of transpiration (reduction in leaf area and decrease in g_s) play a determinant role (Blum, 2011). In addition, maintaining turgor under water deficit can: (i) delay stomatal closure (Mojayad and Planchon, 1994), (ii) maintain the chloroplast volume (Gupta and Berkowitz, 1987; Hubbard et al. 2001), and (iii) reduce leaf wilting (Jones and Turner, 1980; Buckley and Mott, 2002).

Dehydration tolerance (better tolerance to internal cellular water deficit) is a strategy that allows plants to perform physiological functions despite degradation of the water status or to protect cellular water content (Ludlow et al. 1983). This internal water deficit tolerance allows extending the photosynthesis function. Carbon products can then be used for OA rather than for roots growth. Another consequence of the maintenance of the carbon metabolism will be the decrease in the frequency of photo-inhibition period (Maury et al. 1996). In sunflower, there is variability for OA capacities and it depends on the genotype (Maury et al. 2000; Chimenti et al. 2002), scenarios of water deficit (Poormohammad Kiani, 2007b), and the leaf age (Jones and Turner, 1980; Sadras et al. 1993). The solutes essentially involved are inorganic ions (in the vacuole), soluble sugars, polyols, amino acids and organic acids. The

energy cost of such OA is lower in sunflower than in other species such as wheat, since the contribution of inorganic ions is larger (Mccree, 1986; Zhang et al. 1999).

Tolerance to drought is the result of morpho-physiological, biochemical and molecular complex mechanisms. Expression of different genes and accumulation of various osmolytes (OA) that are associated to an effective antioxidant systems are often the main mechanisms of drought tolerance. Many of these mechanisms have been characterized in different plants (Ramanjulu and Bartels, 2002).

2.2.4 Classification of crops by their response to drought

Classification of crop plants base on the physiological response to water deficit and subsequent to drought stress has grouped plants as “isohydric” and “anisohydric” (Tardieu and Simonneau, 1998; Jones, 2013). These characteristics are essential, as they influence the physiological responses observed during drought stress, and can affect the methods best suited to monitoring drought stress (Jones, 2013).

Briefly, the isohydric characteristics of plants resulted from the tight and continuous control of leaf water potential by root-to-shoot signaling through hydraulic and chemical interactions, thus managing water loss through stomata, particularly during the initial onset of water stress (Buckley, 2005). By contrast, plants that display anisohydric characteristics do maintain control over leaf water potential, but it is at diminished rate when compared to isohydric plants. In this condition, soil water content declines as well as leaf water potential until it reaches a threshold at which point stomata begin to regulate water loss (Jones, 2007, 2008).

2.3 Water use efficiency in plant

Carbon dioxide (CO_2) of photosynthesis enters the leaves through the stomata, which also control transpiration. Stomatal closure that keeps the leaf water status reduces photosynthesis and plant production. In a wide range of water deficits compatible with agronomical and physiological activities, the stomatal part is probably the most important (Cornic and Fresneau, 2002). One consequence is that photosynthesis is intrinsically linked to transpiration, and there is no way to circumvent this trade "carbon against water." This exchange is the main limitation of "drought tolerance": we may never build plants that maintain their productivity without a high level of transpiration. However, the ratio of photosynthesis to transpiration, namely leaf WUE, varies with the environmental conditions

and has a significant genetic variability. WUE varies not only with the climate condition but also with the species (Tardieu et al. 2007).

The relationship between water consumption and crop production has become the subject of numerous publications (Ehleringer et al. 1993, Richards et al. 2002, Condon et al. 2004, Blum, 2011). Agronomists were interested in water as a factor of production for crops (Grieu et al. 2008). They showed that there is a significant difference between species and cultivars that need water (Briggs and Shantz, 1913). They began to define the relationship between biomass gain and amount of water consumed: WUE. Then, the need to better understand the determinism of transpiration flow in the field, even in the region, led micro-meteorologists to develop models for determining evapotranspiration (Tardieu et al. 2007). Later, understanding of interactions between carbon gain (photosynthesis) and water use (transpiration), mainly at leaf level, was introduced by the work of de Wit (1958) and Bierhuizen and Slatyer (1965). Currently, all these approaches are developed in order to clarify the relationship between determinism of carbon management and water relations in plants.

WUE could be defined in many ways, depending on the scale of measurement and the units of exchange being considered. All potential definitions will have some measure of water being exchanged for some unit of production (Condon et al. 2004). For physiologists, the basic unit of production could be moles of carbon gained by photosynthesis (A) in exchange for water used in transpiration (E). Thus a physiological definition might equate, at its most basic level, at leaf level, to the instantaneous water use efficiency of leaf gas exchange (A/E) (Ehleringer et al. 1993; Donovan et al. 2007). For agronomists, WUE is defined as the ratio of total plant dry matter produced to total water used over the same period (Tanner and Sinclair, 1983). Besides, for farmers, the unit of production is much more likely to be the yield of harvested product achieved from the water made available to the crop through precipitation and/or irrigation (Condon et al. 2004).

To summarize, the term of WUE in plant can be classified in two levels and scales: (i) leaf level = photosynthetic scale (Farquhar and Richards, 1984; Ehleringer et al. 1993; Bacon, 2004) and (ii) crop level = agronomic scales (Condon et al. 2004; Tuberosa et al. 2007). The terms are provided in Table 2.1 below.

Table 2.1. Several common terms of water use efficiency (WUE)

Level	Time scale	Numerator	Denominator	Equation
Leaf (photosynthetic scale)	Minutes or hours	Net assimilation rate (A) (μmol CO_2 m^{-2} s^{-1})	Transpiration (E) (mmol H_2O m^{-2} s^{-1})	$WUE_i = A/E$
			Stomatal conductance (g_s) (mmol H_2O m^{-2} s^{-1})	$WUE_{ic} = A/g_s$
Crop (agronomic scale)	Weeks to months or growing season	Aboveground biomass (BM) (g or kg)	seasonal evapotranspiration (ET) (ml or l)	WUE = BM/ET
		Grain yield (Y) (g or kg)	seasonal evapotranspiration (ml or l)	WUE = Y/ET

WUEi: instantaneous water use efficiency; WUEic: intrinsic water use efficiency

2.4 Carbon isotope discrimination and water use efficiency

Farquhar et al. (1982) showed the possible use of the discrimination of the heavy isotope of carbon (^{13}C) relative to its light isotope (^{12}C) to directly assess the WUE which can then be easily measured with a mass spectrometer. Approximately 1.1 % of atmospheric CO_2 contains ^{13}C (O'Leary, 1981; Farquhar et al. 1989; Condon et al. 2002). Nevertheless, the molar abundance ratio of $^{13}C/^{12}C$ in plant tissues usually is less than that in atmospheric CO_2 because of discrimination against the 'heavier' ^{13}C (lower reactivity) during photosynthesis: firstly, during diffusion of CO_2 into the leaf through the stomata and, secondly, during the first key step in CO_2 fixation by C_3 plants, catalyzed by the enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase) (O'Leary, 1981; Farquhar et al. 1982; Farquhar and Richards, 1984; Farquhar et al. 1989).

Carbon isotope discrimination (CID) or $\Delta^{13}C$ is a measure of the ratio of the stable isotopes of carbon ($^{13}C/^{12}C$) in plant material relative to the value of the same ratio in the atmosphere (Farquhar et al. 1989; Condon, 2004). Since the pioneering work of Farquhar and colleagues (Farquhar et al., 1982; Farquhar and Richards, 1984), it has subsequently been demonstrated, for several C_3 species, that variation in CID closely reflects variation in WUE. As predicted by theory (reviewed in Farquhar et al. 1982; Farquhar et al. 1989; Hall et al. 1994; Condon and Hall, 1997; Condon, 2004): WUE and CID should be negatively related. Indeed, when stomatal closure increase, transpiration decreases, discrimination against ^{13}C decreases, and WUE increase.

Currently CID is widely used as an indirect assessment of WUE in C_3 crops under water-limited conditions. Extensive studies in C_3 species have been reported and have confirmed the negative relationship between CID and WUE (Hubick et al. 1986; Condon et al. 1990; Lauteri et al. 1993; Wright et al. 1994; Virgona and Farquhar, 1996; Scartazza et al. 1998; Lambrides et al. 2004; Impa et al. 2005; Misra et al. 2010; Roel et al. 2011). This relationship in C_3 plants has opened up the prospect of utilizing differences in ^{13}C discrimination (CID) for selecting crops that have high WUE under specific environments.

2.5 Genetic analysis of water use efficiency, carbon isotope discrimination and plant-water relation traits

Understanding the genetic basis of WUE or CID is important for crop improvement (breeding) under water-limited environments (Chen et al. 2011). It has been shown that with adequate attention to sampling strategies, CID is a highly heritable trait that is relatively easy to manipulate in breeding populations (Condon and Richards, 1992; Rebetzke et al. 2002). All these observations indicate CID as a potential candidate for use in breeding for greater agronomic WUE (Condon, 2004). In sunflower, genetic quantitative study has been conducted to analyze the genotypic variability of WUE and the potential use of leaf CID as an indicator to determine WUE (Lambrides et al. 2004). However, determination and improvement of WUE through conventional breeding programs is not practical because of the complexity and difficulty of measuring WUE of a large number of breeding lines under field conditions (Mian et al. 1996; Nguyen et al. 1997; Ober et al. 2005). Thus, there is a need for finding an alternative to the conventional approach for the improvement of WUE of field crops. Indirect selection for improved WUE through molecular-markers approaches conditioning WUE for crops may prove to be a useful approach in this respect (Mian et al. 1998), since quantitative traits such as WUE and CID are generally under considerable environmental influence, and are governed by quantitative trait loci (QTL) (Hall et al. 1994; Juenger et al. 2005; Brendel, 2008).

QTL provides the opportunity to compare whether different traits have a common genetic basis (Tanksley, 1993; Li et al. 1995). Further, QTL mapping: (i) is based on the principle that genes and markers segregate via chromosome recombination (called crossing-over) during meiosis (i. e. sexual reproduction), thus allowing their analysis in the progeny (Paterson, 1996) and (ii) usually provides a starting point for statistically identifying the chromosomal regions contributing to variation of agronomical traits in breeding programs (Zhang, 2007;

Chen et al. 2011). Phenotypic correlations are commonly used to associate markers with traits and to genetically dissect complex traits into Mendelian factors. Once markers that are tightly linked to genes or QTL of interest have been identified, breeders may use these markers as diagnostic tools to identify lines carrying the genes or QTL in a marker-assisted selection (MAS) program (Liu, 1998). The selected QTL should account for the largest proportion of the phenotypic variance for the target trait. So by using larger population sizes and a greater number of markers, more tightly-linked markers can be identified in high resolution mapping (Mohan et al. 1997).

In general, polygene controlling WUE and CID are multiple genes each with small effects, implies that several QTL must be manipulated simultaneously to obtain a major impact (Cattivelli et al. 2008). It is preferable to target QTL with a major effect that is consistent across environments and populations and also independent of the genetic background (Rebetzke et al. 2008). From this context, the ultimate goal of QTL mapping is to transfer QTL of WUE and CID into elite breeding lines to improve their performance when drought happens (Shinozaki and Yamaguchi-Shinozaki, 2007; Chen et al. 2011).

The QTL mapping of WUE in crop plants is rarely reported in the literature. Four QTL associated with WUE have been identified in soybean (Mian et al. 1996). The inheritance of WUE has been studied using simple sequence repeat (SSR) markers in alfalfa (Julier et al. 2010). In contrast, QTL mapping of CID has been reported by numerous authors. The first QTL identified for CID was reported in tomato by Martin & Nienhuis (1989) and subsequently QTL for CID have been reported in *Arabidopsis thaliana* (Hausmann et al. 2005), barley (Ellis et al. 1997; Diab et al. 2004), cotton (Saranga et al. 2001), rice (Takai et al. 2006; This et al. 2010), soybean (Specht et al. 2001) and wheat (Rebetzke et al. 2008; Peleg et al. 2009). Nevertheless, QTL of WUE and CID in sunflower have never been reported.

Besides, the genetic control of the main traits controlling water flow from soil to the atmosphere (plant-water relation traits, i.e. transpiration control, water extraction capacity and dehydration tolerance) remains poorly understood. There have been no reports of QTL identification for threshold of the FTSW (FTSWt; transpiration control). Marguerit et al. (2012) reported the results of QTL analysis for the acclimation of transpiration rate to water deficit in grapevines: calculated values of NTR when FTSW reached 60% ($NTR_{FTSW60\%}$),

40% ($NTR_{FTSW40\%}$) and 20% ($NTR_{FTSW20\%}$). They also reported QTL mapping for total transpirable soil water (TTSW; water extraction capacity), however, QTL mapping for TTSW in sunflower was never reported. In addition, despite OA is receiving increasing recognition as major mechanism of dehydration and drought tolerance (Flower and Ludlow, 1986; Zhang et al. 1999; Serraj and Sinclair, 2002), and genetic analysis (or QTL mapping) for OA has been reported by numerous authors in a wide range of crops (Morgan, 1984; Teulat et al. 1998; Saranga et al. 2004), QTL mapping for OA associated with dehydration and drought tolerance in sunflower is rarely explored (Poormohammad Kiani et al. 2007b).

3 MATERIALS AND METHODS

3.1 Plant material

Experiments of this Thesis are based on 150 recombinant inbred lines (RILs), including their parents (XRQ and PSC8), except for the experiment of section 4.3 and 4.4 only consisting of four RILs: RIL 043, RIL 127, RIL 149, and RIL 200.

The XRQ and PSC8 are parental lines of the "INEDI" RIL population developed by INRA[3], and behaved differently in response to water use (Rengel et al. 2012). The INEDI RIL population was obtained by single seed descent, self-pollination to at least F8 (Vincourt et al. 2012). In addition, the list of all genotypes (150 genotypes) is provided in Appendix 1.

3.2 Growth conditions and experimental setup

3.2.1 Experiment in greenhouse

Plants for four experiments across two different years (Section 4.1, 4.2 and 4.4), conducted on two drought scenarios and five levels of SWC, were grown in a greenhouse at the INRA Auzeville station, Toulouse, France (43°31'46,94" N; 1°29'59,71" E). Greenhouse air temperature was set at 25/18 $\pm$ 2^0C (day/night) and relative humidity (RH) was about 55-75 $\pm$ 5%.

Three seeds per genotype were sown in a pot (volume: 2 liters) at the beginning of the experiments. Ten to eighteen days after sowing (DAS), depending experiments, the most vigorous plant (based on morphological criteria) in each pot was selected by cutting down the two others. Each pot was then covered with a 3 mm layer of polystyrene sheet with a hole in the middle to allow normal plant growth, thus reducing the evaporation of water from the soil surface.

The pots contained a mixture of 50% soil (collected from the field), 30% organic matter and 20% sand, except for one experiment (section 4.4) where pots were filled with soil extracted from the field and sand in equal proportions. The pots were arranged on 100 balances (maximum capacity 30 kg, precision 2 g, model SXS, GRAM, Spain; Fig. 3.1) that were connected by interface

[3] Institut national de la recherche agronomique

wireless communication to a computer with installed software (ENSAT 1.07T, developed by Pesage du Sud Ouest, Launaguet, France).

Figure 3.1 Pots were arranged on the balances in the greenhouse.

Drought scenario 1 (experiment in 2011; using 150 genotypes)

A randomized complete block design with three replicates was used for the progressive water stress treatments (three replicates X 150 genotypes = 450 plants; called WS). There was another replicate (150 plants) that was considered as a well-watered treatment, called WW.

At 1 day after emergence (DAE), 17 DAS, all 600 pots were watered to field capacity, corresponding to 39.5% of soil water content (SWC). These 600 pots (WW and WS) were kept without irrigation until 17 DAE. Starting at 17 DAE, when genotypes reached around 23% of SWC, we irrigated the WW treatment to 30% of SWC and we maintained this SWC by daily irrigation (Fig. 3.2) The WS treatment was kept without irrigation until harvest (Fig. 3.4). This experiment was called Exp. 2011 or drought scenario 1.

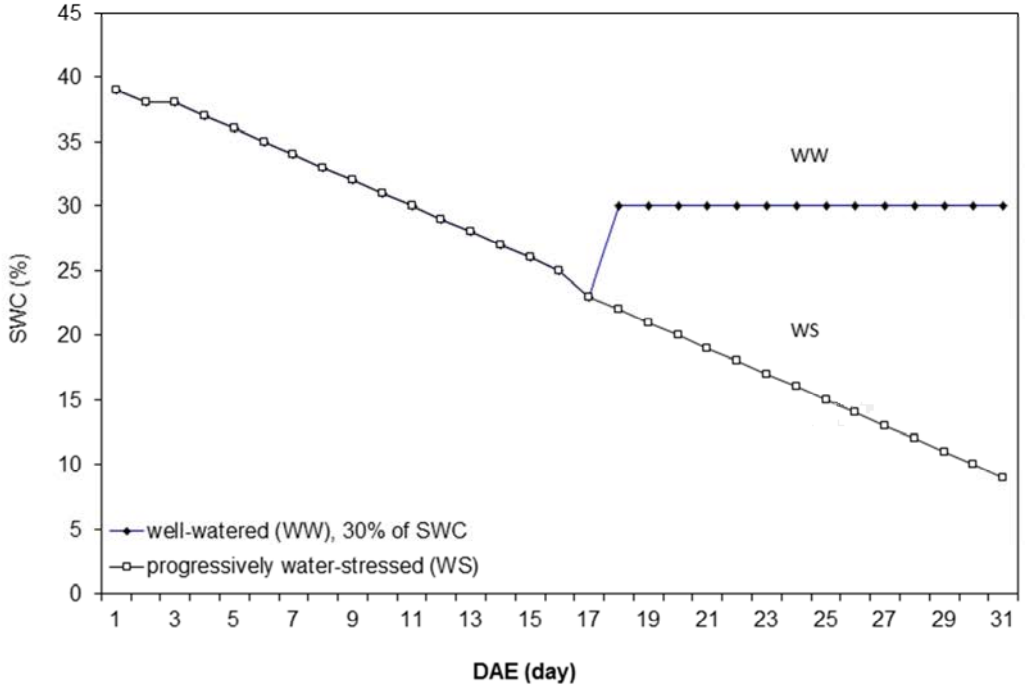

Figure 3.2 Principles of the water treatments used in the experiment

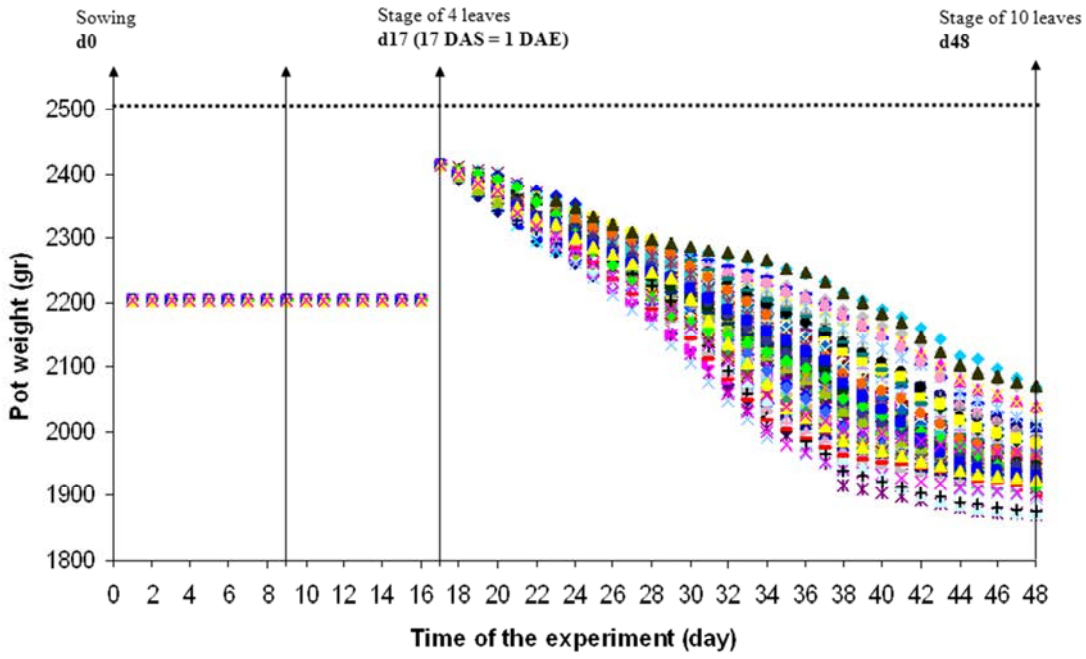

Figure 3.3 Pot weight evolution of water-stressed (WS) treatment

Drought scenario 2 (experiment in 2012; using 150 genotypes)

A randomized complete block design with two treatments and two replicates was performed (300 pots per treatment). Treatments consisted of two levels of stable SWC which was imposed: well-watered (30% of SWC, namely WW) and water-stressed (16% of SWC, namely WS). At 1 DAE (19 DAS), stable water contents corresponding to 30% of SWC (WW) and 16% of SWC (WS) were maintained for 23 days (Fig. 3.4). This experiment was called Exp. 2012 or drought scenario 2.

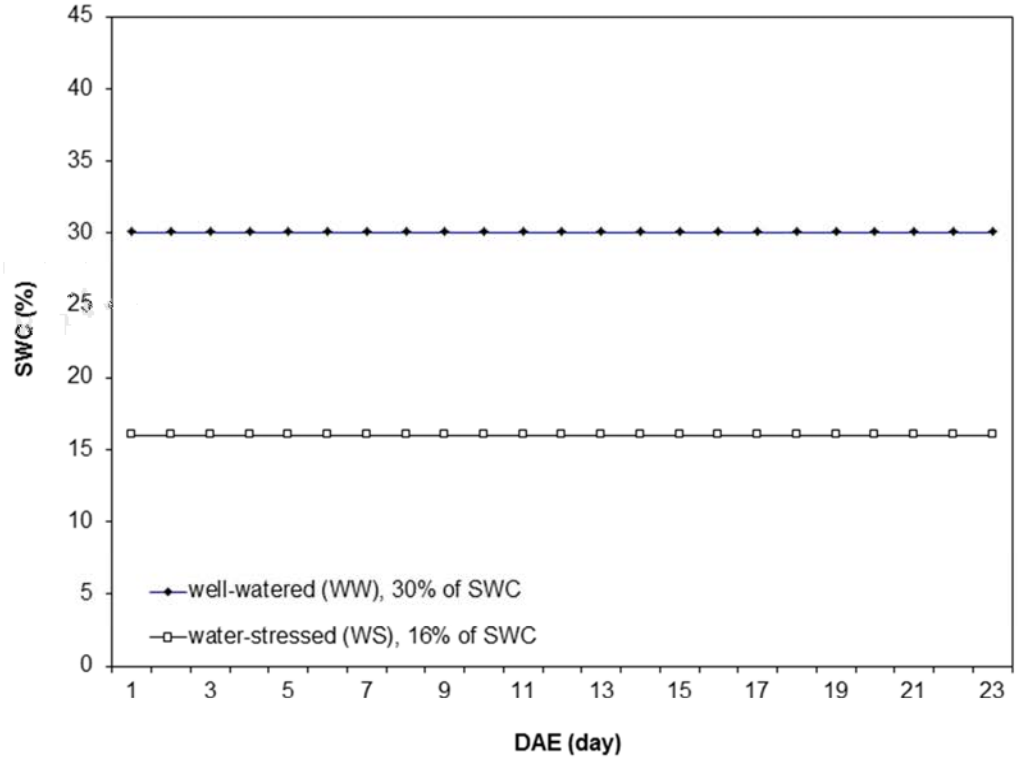

Figure 3.4 Principles of the water treatments used in the experiment

Five levels of SWC (two experiments in 2012; using four genotypes)

The experiments were arranged in a randomized complete block design with four RILs, five water treatments and five replicates. In experiment 1 (Exp. 1; spring 2012), water treatments were applied consisting in five levels of SWC: 35%, 23%, 21%, 18% and 16%. In experiment 2 (Exp. 2; autumn 2012), water treatments consisted of five levels of SWC: 25%, 20%, 16%, 13% and 10%. Starting at 21 DAS, the plants were subjected to different water treatments (Fig. 3.5).

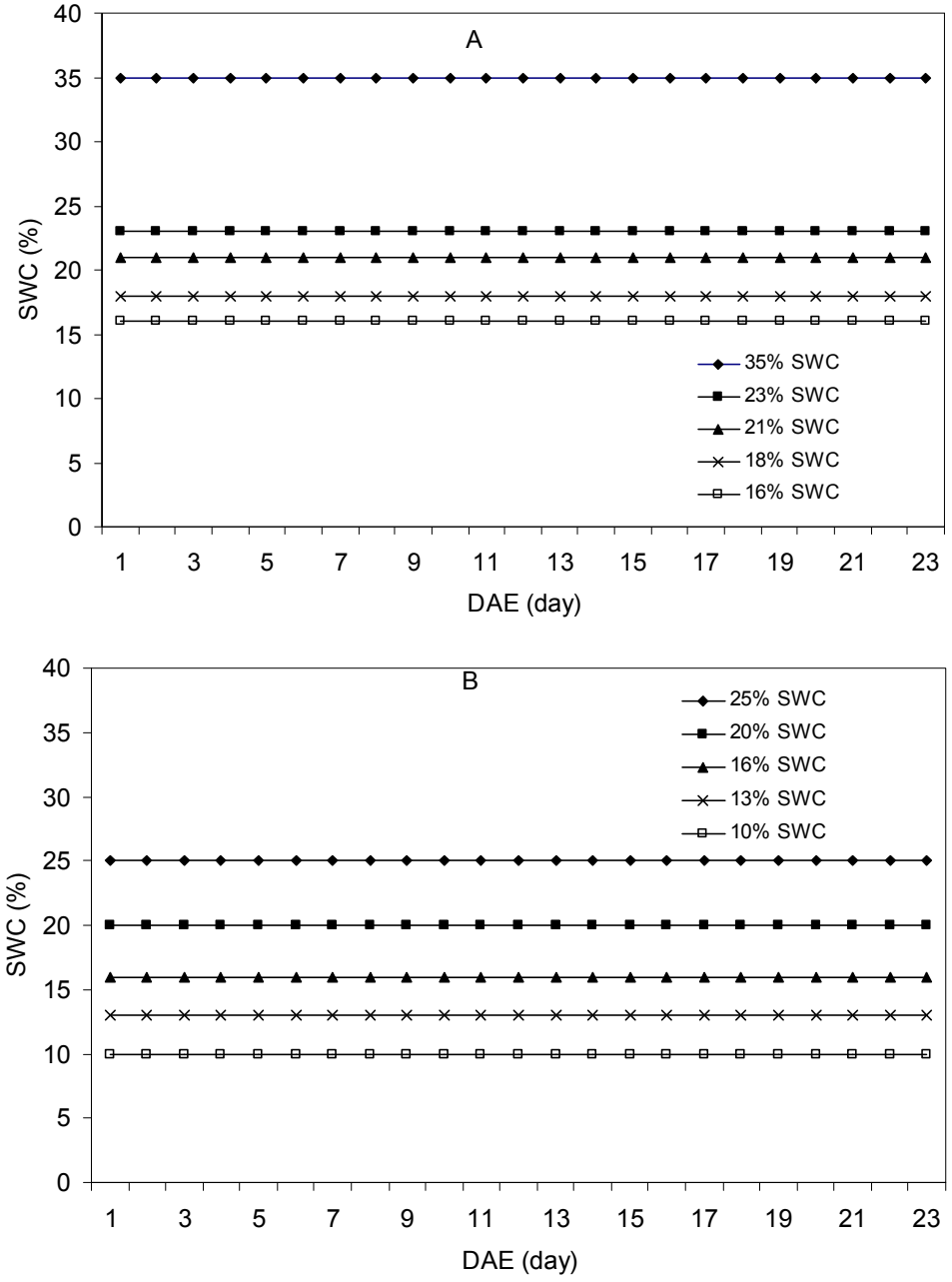

Figure 3.5 Five levels of soil water content (SWC) maintained stable from emergence to harvest in Exp. 1 (A) and in Exp. 2 (B)

3.2.2 Experiment in growth chamber ("*phytotron*")

Plants for the experiment of "root hydraulic conductivity and contribution of aquaporins (AQPs) to water uptake" (section 4.3) were grown in a growth chamber (25°C/20°C in day/night) (Fig.

3.6) under 14 h of light, 200 µmol m^{-2} s^{-1} photosynthetically active radiation at leaf level (neon type: L 58 W/77, 2250 lm, Fluora, Germany), and 50% RH in 250 ml pots. The plants were arranged in a randomized complete design with four RILs and four replicates without water-stressed treatment (all the plants were irrigated daily). Seedlings were grown in 250 ml glass pots of sand which could be easily washed and saturated with solution and then introduced into the pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

To minimize the effects of heterogeneity within the growth chamber, the pots were rotated every week. For pressure-flow experiments, upon harvest, pots were washed three times (3 x 50 ml) to saturation with water for the control treatment and $HgCl_2$ solution (500 µM) for the inhibited treatment. In addition, the experiment was repeated three times.

Figure 3.6 Examples of plants in the growth chamber

3.3 Phenotypic analysis: Trait measurements

3.3.1 Water use efficiency and carbon isotope discrimination

In general, for all experiments, WUE at plant level was determined as the ratio of biomass (BM) to cumulative water transpired (CWT). For Exp. 2011 (drought scenario 1), there were two methods to determine WUE. The first was the total water use efficiency, WUE_{T2011}, calculated by

dividing the BM by the CWT_{31d} ($WUE_{T2011} = \frac{BM}{CWT_{31d}}$); (Equation 1). CWT_{31d} is the total of plant transpiration during 31 days (from 1 to 31 DAE). The second calculation of WUE was made during the period when the two treatments differed in their soil water content (WW and WS), from 17 to 31 DAE, and called WUE_{E2011} (water use efficiency "estimation"). WUE_{E2011} was calculated by dividing the "estimated biomass" (BM_E), by the CWT_{15d}, calculated from 17 to 31 DAE ($WUE_{E2011} = \frac{BM_E}{CWT_{15d}}$); (Equation 2). The BM_E was calculated as follows.

$$BM_E = BM_{31} - BM_{17} \quad \text{(Equation 3)}$$

where BM_{17} is the biomass estimated at 17 DAE. The BM_{17} was calculated as follows.

$$BM_{17} = \left[\frac{LA_{17}}{LA_{31}}\right] \times BM_{31} \quad \text{(Equation 4)}$$

where LA_{17} and LA_{31} are the leaf areas measured on 17 and 31 DAE, respectively.

WUE at leaf level, intrinsic WUE, was calculated as the ratio of A to g_s (see table 2.1). This calculation was only done in Exp. 2 of the experiments that use four RILs (section 4.4). The values of A to g_s were obtained from leaf gas exchange measurements. These measurements were made with a portable Li-6400 (Li-Cor, Lincoln, NE, USA) between 09:00 and 12:00 (Central European Time) from 19 to 21 DAE. All the measurements were conducted on a fully-expanded leaf (one per plant) under 1500 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and 40 Pa CO_2 partial pressure. Leaf temperature was maintained at 25 ± 2^0C and RH was 50%.

For carbon isotope discrimination measurement, oven-dried leaves (including petioles) of each plant were ground into a homogeneous fine powder and 2-3 mg subsamples were weighed and placed in capsules (Elemental Microanalysis, UK) to analyze its carbon isotope composition ($\delta^{13}C$) by using a continuous flow Isotope Ratio Mass Spectrometer (IRMS) at UC Davis Stable Isotope Facility (California, USA). CID (or $\Delta^{13}C$) was then calculated as described by Farquhar and Richards, (1984), and Farquhar et al. (1989):

$$\Delta^{13}C(‰) = \frac{Rair}{Rplant} - 1 = \frac{\delta^{12}C_{air} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}/1000} \quad \text{(Equation 5)}$$

where *R*air and *R*plant refer to $^{13}C/^{12}C$ ratios of the atmosphere and plant samples, respectively.

The ratio of $^{13}C/^{12}C$ in a sample of plant is converted to $\delta^{13}C$ which is commonly compared with a reference material, the belemnite carbonate standard from the Pee Dee Formation (PDB) in South Carolina (Craig, 1953; O'Leary, 1981; Farquhar et al. 2001). On the PDB scale, the $\delta^{13}C$ value for free atmospheric CO_2 is approximately -8‰ (Farquhar et al. 1989).

3.3.2 Transpiration

Transpiration, called water transpired (WT), for each plant was estimated every day from the difference in the pot weight. Total transpiration (CWT) for each plant was determined at the end of the experiment by accumulating daily WT.

3.3.3 Biomass

At the end of the experiments, the above-ground parts of the plants were harvested. Stems and leaves were oven dried at 80^0C for 48h until they reached constant mass to determine total dry aerial biomass (BM).

3.3.4 Plant-water relation traits

The measurement of plant-water relation traits was mainly focused on Exp. 2011. Three main traits controlling plant-water relation traits include (i) control of transpiration, expressed as threshold of fraction of transpirable soil water (FTSWt), (ii) water extraction capacity, expressed as total transpirable soil water (TTSW), and (iii) dehydration tolerance, expressed as osmotic adjustment (OA) by measuring osmotic potential at full turgor at WW (OP_ww) and WS (OP-ws) conditions. The measurement of all plant-water relation traits was clearly explained in detail below.

Control of transpiration

First of all, the soil water status in the pots for each plant was calculated by soil water content (SWC) as follows.

$$SWC = \frac{\left(PW_d - PW_{wp}\right)}{PW_{wp}} \quad \text{(Equation 6)}$$

where PW_d was the pot weight on a given date and PW_{wp} was final pot weight at wilting point.

In this experiment, we normalized SWC by using fraction of transpirable soil water (FTSW), as has been proposed by Ritchie (1981), and Sinclair and Ludlow (1986). The daily value of FTSW

was calculated as the ratio between the amount of transpirable soil water still remaining in the pot and TTSW:

$$FTSW = \frac{\left(PW_d - PW_{wp}\right)}{TTSW} \quad \text{(Equation 7)}$$

Transpiration of WS and WW plants was used to determine normalized transpiration ratio (NTR). Firstly, transpiration rate was calculated by dividing the transpiration of each individual plant of a given genotype by the leaf area (LA) of plant of that genotype. Secondly, the transpiration rate was normalized by dividing each transpiration rate value of WS plant (for each replicate) by transpiration rate value of WW plant. This second normalization gave NTR, which accounted for plant to plant variation in transpiration within each genotype

Finally, the measurement of plant response to water deficit (traits related to the control of transpiration) used a regression approach to model individual plant response. The parameters from these models were used as quantitative traits in the association analysis. Two traits were estimated by using break-linear models: (i) FTSWt, the threshold of transpirable soil water (FTSW) at which the plant transpiration rate (NTR) began to decline, Equation 8, and (ii) SWCt, the value of soil water content (SWC) when the plant transpiration (NTR) rate was null, Equation 9 and 10.

(i)

If $x < a$, $NTR = \frac{0.9}{a} \times x + 0.1$

else, $NTR = 1$ (Equation 8)

where x was FTSW, and a was FTSWt.

(ii)

If $x < a$, $NTR = \frac{1-b}{a} \times x + b$

else, NTR =1 (Equation 9)

where x was SWC, a was SWCt, the x-intercept was computed as:

$$x0 = -b \times \frac{a}{1-b} \quad \text{(Equation 10)}$$

Water extraction capacity

For each pot, at the end of the experiment, TTSW was calculated as follows.

$$TTSW\,(ml) = PW_{fc} - PW_{wp} \quad \text{(Equation 11)}$$

where PW_{fc} was initial pot weigh at field capacity.

Osmotic potential and adjustment

The leaves samples were taken when plants began to be wilt (it was two days before the wilting point was reached). These samples were the half of fully expanded leaf for each individual plant (without petiole).

Before measuring OP, the leaves samples were rehydrated in distilled water during 24 h at 4^0C in a dark room. This was aimed to make OP at full turgor: called OP_ww and OP_ws for the WW and WS plants, respectively. After rehydration, the osmotic values of leaves samples were measured on expressed sap using 10 µl aliquots placed in an osmometer (Wescor, model 5520, Logan, UT, USA) calibrated with manufacturer solutions. OP was then determined by calculating the osmometer reading (in mmol kg^{-1}) using the Van't Hoff relation:

$$OP(MPa) = \frac{-RTd \times c}{1000} \quad \text{(Equation 12)}$$

where R was gas constant, T was temperature in Kelvin, *d* was density of water at temperature T, and *c* was concentration of osmotically-active solutes, given by the osmometer. OA was then determined using the following equation:

$$OA(MPa) = OPww - OPws \quad \text{(Equation 13)}$$

where the value of OP_ws was represented by the mean of the replicates.

3.3.5 Root measurement

The measurement of roots properties and hydraulics was only conducted on experiment in growth chamber (Section 4.3). In order to measure root hydraulics (conductance, conductivity and contribution of AQPs), pressure-induced sap rates were determined on six-week-old sunflower seedlings, when above-ground parts were 15-20 cm high.

Before measuring the sap flow (Jv), the plants were washed with water (for control treatment) and with $HgCl_2$ (for inhibition treatment). Following this washing, the above-ground part was cut

off with a razor blade just below the cotyledonary leaves (40-50 mm from the base). Pots with whole root systems were placed in a stainless steel pressure chamber. Excised stems were sealed into the lid of the chamber through a silicone gasket so that part of the stem protruded and chamber pressure was gradually increased. Water expressed from each cut stem was collected using an Eppendorf tube containing dry cotton wool. The amount of sap was determined by weighing the tube before and after collecting the water. The Jv, expressed as the quantity of water exuded from the cut stems, was monitored every 5 min for at least 45 min after it had reached a constant rate (less than 25 min).

Upon completion of the exudation experiments, root fresh weight was weighed. Then, properties i.e. root length, root surface area and volume of fine roots of each root system were determined with an image analyzer WinRHIZO 2007d (Regent Instruments, Quebec, Canada). Fine roots are the smallest diameter class (0 – 0.5 mm). Finally, roots were oven dried at 80^0C for 48h until they reached constant mass to determine root dry weight

The Jv previously obtained was then used to define (i) the whole root hydraulic conductance (root L_0) calculated as the sap flow rate per unit of pressure (µL s^{-1} MPa^{-1}) and (ii) the root hydraulic conductivity (root Lp_r) calculated as the sap flow rate per unit of root surface and per unit of pressure (m s^{-1} MPa^{-1}). In addition, the contribution of AQPs to root Lp_r (AQPs involvement) was determined by expressing as the relative decrease in root Lp_r induced by $HgCl_2$ treatment.

3.3.6 Other traits measurement

Other traits measurements in the greenhouse experiments consisted of morphological traits measurements including plant height (PH), leaf number (LN) and leaf area (LA). The measurement of PH and LN was done at the end of experiment (before harvesting the plants). For LA, due to numerous plants in the experiment, LA of the plants was estimated by using computer image analysis system, winFOLIA (Regent Instruments, Quebec, Canada). The image of leaves was taken by a digital camera (Canon, eos400d).

3.3.7 Statistical analysis

For all experiment, the software of statistical package PASW statistics 18 (IBM, New York, USA) was used to analyze genotype and replicate effects by analysis of variance (ANOVA) and to estimate phenotypic correlation by Pearson's correlation. Means of the traits were compared using a Student-Newman-Keuls (SNK) test ($P < 0.05$), except, for the FTSW and SWC threshold (FTSWt and SWCt) analysis, R software (R Development Core Team, 2012) was used. Each NTR value was plotted to a corresponding FTSW and SWC values. FTSWt and SWCt where NTR initiated its decline were determined using a plateau regression.

3.4 Genetic analysis

3.4.1 Heritability

The broad sense heritability (h^2) was computed from the estimates of genetic (σ^2g) and residual (σ^2e) variances derived from the expected mean squares of the ANOVA as follows.

$$h^2 = \frac{\sigma^2 g}{\left(\sigma^2 g + \frac{\sigma^2 e}{r}\right)}$$ (Equation 14), where r was the number of replicates.

3.4.2 Genetic map construction

The genetic map consisted of 2610 markers located on the 17 LG for a total genetic distance of 1863.1 cM and grouped on 999 different loci. The gDNA from the INEDI RILs population obtained from the cross between XRQ and PSC8 lines (210 samples) were genotyped with the Infinium array. All genotyping experiments were performed by Integragen (IntegraGen SA, Genopole Campus 1 - Genavenir 8, 5 rue Henri Desbruères, 91000 Evry, France.) and the genotypic data were obtained with the Genome Studio software (Illumina) with automatic and manual calling. A set of 9832 SNPs were used to produce an Infinium HD iSelect BeadChip (Infinium). These SNPs were selected from either genomic re-sequencing or transcriptomic experiments. From the 9832 SNPs, 2576 were polymorphic between XRQ and PSC8. We used CarthaGène v1.3 (De Givry et al. 2005) to build the genetic maps. We added the genotypic data of markers from a consensus map (Cadic et al. 2013) to assign the Infinium SNPs to the appropriate LG.

3.4.3 QTL mapping

QTL mapping was carried out using MCQTL, software for QTL analysis (http://carlit.toulouse.inra.fr/MCQTL/). MCQTL package is comprised of three software applications. The first component, TranslateData reads data from MAPMAKER (Lincoln et al. 1993) like files. The second component, ProbaPop computes QTL genotype probabilities given marker information at each chromosome location for each family and stores them in XML formatted files. The last component, Multipop builds the pooled model using the genotype probabilities, computes Fisher tests and estimates the model parameters (Jourjon et al. 2005). Significant thresholds ($P < 0.05$) for QTL detection were calculated for each dataset using 1000 permutations (Churchill & Doerge, 1994) and a genome-wide error rate of 0.01 (Type I error). The corresponding type I error rate at the whole-genome level was calculated as a function of the overall number of markers in the map and the number of markers in each linkage group.

4 RESULTS AND DISCUSSIONS

4.1 Genetic control of water use efficiency and leaf carbon isotope discrimination in sunflower (*Helianthus annuus* L.) subjected to two drought scenarios

Afifuddin Latif Adiredjo[1,2], Olivier Navaud[3], Stephane Muños[4,5], Nicolas Langlade[4,5], Thierry Lamaze[3*], Philippe Grieu[1*]

1 Université de Toulouse, INP-ENSAT, UMR1248 AGIR (INPT-INRA), Castanet-Tolosan, France, 2 Brawijaya University, Faculty of Agriculture, Department of Agronomy, Plant Breeding Laboratory, Malang, Indonesia, 3 Université de Toulouse, UPS-Toulouse III, UMR5126 CESBIO, Toulouse Cedex 9, France, 4 INRA, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, Castanet-Tolosan, France, 5 CNRS, Laboratoire des Interactions Plantes-Microorganismes(LIPM), UMR2594, Castanet-Tolosan, France, *PhD supervisors of the first author

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Correspondence

Philippe Grieu

Université de Toulouse, INP/ENSAT, UMR 1248 AGIR, BP BP 32607, 31326 Castanet-Tolosan, France

Tel.: +33(0)534323878

Fax.: +33(0)534323901

Email: grieu@ensat.fr

Abstract

High water use efficiency (WUE) can be achieved by coordination of biomass accumulation and water consumption. WUE is physiologically and genetically linked to carbon isotope discrimination (CID) in leaves of plants. A population of 148 recombinant inbred lines (RILs) of sunflower derived from a cross between XRQ and PSC8 lines was studied to identify quantitative trait loci (QTL) controlling WUE and CID, and to compare QTL associated with these traits in different drought scenarios. We conducted greenhouse experiments in 2011 and 2012 by using 100 balances which provided a daily measurement of water transpired, and we determined WUE, CID, biomass and cumulative water transpired by plants. Wide phenotypic variability, significant genotypic effects, and significant negative correlations between WUE and CID were observed in both experiments. A total of nine QTL controlling WUE and eight controlling CID were identified across the two experiments. A QTL for phenotypic response controlling WUE and CID was also significantly identified. The QTL for WUE were specific to the drought scenarios, whereas the QTL for CID were independent of the drought scenarios and could be found in all the experiments. Our results showed that the stable genomic regions controlling CID were located on the linkage groups 06 and 13 (LG06 and LG13). Three QTL for CID were co-localized with the QTL for WUE, biomass and cumulative water transpired. We found that CID and WUE are highly correlated and have common genetic control. Interestingly, the genetic control of these traits showed an interaction with the environment (between the two drought scenarios and control conditions). Our results open a way for breeding higher WUE by using CID and marker-assisted approaches and therefore help to maintain the stability of sunflower crop production.

Keywords: water use efficiency, carbon isotope discrimination, genetic control, drought, sunflower

Introduction

Water use efficiency (WUE) as a breeding target can be defined as the ratio of biomass production to water consumption. Breeding for WUE and drought-resistant crop varieties has been a critical area of agricultural research worldwide [1-3]. Substantial efforts have been devoted to identifying and selecting for morphological and physiological traits that increase WUE and yield under rain-fed conditions [2,4-5]. In field conditions, water consumption is usually difficult to determine. Nevertheless, WUE can be represented by measuring leaf carbon isotope discrimination (CID) [6-7]. Because the CID has been demonstrated to be a simple but reliable measure of WUE, the negative correlation between them has been used as an indirect method in selection to improve WUE [8-10]. The principle mechanisms underlying the variation of CID act through variation in the intercellular CO_2 concentration (c_i) maintained in leaves [6]. The value of c_i is determined through the coordinated regulation of carboxylation capacity (photosynthesis) and stomatal control of leaf diffusive conductance (transpiration regulation) [6-7].

Genetic variation underlying quantitative traits, such as WUE and CID, that are generally under considerable environmental influence, is governed by quantitative trait loci (QTL) [11-14]. QTL mapping provides a starting point in breeding programs [15-16] and for cloning of the causal mutations by fine mapping.

QTL mapping of WUE is rarely reported. Four QTL associated with WUE have been identified in soybean [17]. The inheritance of WUE has been studied using simple sequence repeat (SSR) markers in alfalfa [18]. In contrast, QTL mapping of CID has been reported by numerous authors. The first QTL identified for CID was reported by Martin and Nienhuis [19]. These authors identified four QTL associated with CID in tomato. Since that time, QTL for CID have been identified across a wide range of species, for example in cotton [20], rice [21], barley [22], *Arabidopsis* [23], and in wheat [24]. However, to our knowledge, QTL of WUE and CID in sunflower have never been reported.

Most of the work identifying QTL of WUE and CID has been done in well-watered conditions, with only one study in a drought situation. There is no report on the QTL identification of WUE and CID of crops subjected to different scenarios of water deficit establishment.

The objectives of the present study are to identify QTL controlling WUE and CID in a population of RILs of sunflower, and to compare QTL associated with these traits in a dual drought scenario: (i) a progressively water-stressed establishment and (ii) a stable water deficit treatment. We are interested in providing new insights into the genetic architecture of WUE and CID, and in contributing to the potential of sunflower breeding by improved WUE.

Materials and Methods

Plant materials

A population of 150 recombinant inbred lines (RILs) was used in two experiments. A population of these RILs was named INEDI and was obtained by single seed descent (self-pollination to at least F8) from a cross between XRQ and PSC8 [25].

Experiments and trait measurements

Two experiments were conducted in spring 2011 (Exp. 2011) and in spring 2012 (Exp. 2012) under quite similar weather conditions. Plants were grown in a greenhouse at the INRA Auzeville station, Toulouse, France (43°31'46,94" N; 1°29'59,71" E). Greenhouse air temperature was set at 25/18 $\pm$ 2^0C (day/night) and relative humidity was about 55-75 $\pm$ 5%.

Three seeds per genotype were sown in a pot (volume: 2 liters) at the beginning of the experiments. The pots contained a mixture of 50% soil (collected from the field), 30% organic matter and 20% sand. These pots were arranged on 100 balances (maximum capacity 30 kg, precision 2 g, model SXS, GRAM, Spain), with six pots per balance (total pot number in greenhouse was 600). Each pot was then covered with a 3 mm layer of polystyrene sheet with a hole in the middle to allow normal plant growth, thus reducing the evaporation of water from the soil surface. Throughout the experiments, the amounts of water in the pots were determined by weighing the pots every day. This weighing recorded the amount of daily water loss, corresponding to the daily transpiration of the plants. For each pot, at the end of the experiment, cumulative daily transpiration was called CWT (the cumulative water transpired). Biomass was separated into leaves and stems at harvest. Total dry aerial biomass (BM) was obtained after drying at 80^0C for 48 h. WUE was determined at the end of the experiment, defined as the ratio of BM to CWT. In addition, a dual drought scenario strategy for the two experiments (explained in detail below) was studied.

Experiment conducted in 2011: scenario of progressive water stress

A randomized complete block design with three replicates was used for the progressive water stress treatments (three replicates X 150 genotypes = 450 plants; called WS). There was another replicate (150 plants) that was considered as a well-watered treatment, called WW.

At 1 day after emergence (DAE), 17 days after sowing (DAS), all 600 pots were watered to field capacity, corresponding to 39.5% of soil water content (SWC). These 600 pots (WW and WS) were kept without irrigation until 17 DAE (Fig. 1A). In these conditions, stomatal conductance of the plant was still not affected. We calculated that stomatal conductance started to decrease at an average SWC of about 21% (unpublished data).

Starting at 17 DAE, when genotypes reached around 23% of SWC, we irrigated the WW treatment to 30% of SWC and we maintained this SWC by daily irrigation. The WS treatment was kept without irrigation until harvest (during 15 days).

Two determinations of WUE were made. The first was the total water use efficiency, WUE_{T2011}, calculated by dividing the BM by the CWT_{31d}. CWT_{31d} is the cumulative water transpired during 31 days (from 1 to 31 DAE). The second calculation of WUE was made during the period when the two treatments differed in their soil water content (WW and WS), from 17 to 31 DAE, and called WUE_{E2011} (water use efficiency "estimation"). WUE_{E2011} was calculated by dividing the "estimated biomass" (BM_E), by the CWT_{15d}, calculated from 17 to 31 DAE. $BM_E = BM - BM_{17}$, where BM_{17} is the biomass estimated at 17 DAE. In addition, the BM_{17} was calculated as follows: $BM_{17} = (LA_{17}/LA_{31})$ X BM, where LA_{17} and LA_{31} are the leaf areas measured on 17 and 31 DAE, respectively.

Experiment conducted in 2012: scenario of stable SWC

A randomized complete block design with two treatments and two replicates was performed (300 pots per treatment). Treatments consisted of two levels of stable SWC which was imposed: well-watered (30% of SWC, namely WW) and water-stressed (16% of SWC, namely WS) (Fig. 1B).

At 1 DAE (19 DAS), stable water contents corresponding to 30% of SWC (WW) and 16% of SWC (WS) were maintained for 23 days (Fig. 1B). WUE was calculated by dividing the BM by the CWT_{23d} (WUE_{T2012}), where CWT_{23d} is the cumulative water transpired during 23 days (from 1 to 23 DAE).

Determination of carbon isotope discrimination (CID)

Carbon isotope composition (δ) was calculated relative to the international Pee Dee Belemnite (PDB) standard [26]: $\delta_{plant} = (R_{sa} - R_{sd})/R_{sd}$ X 1000 [‰] where R_{sa} and R_{sd} are the ^{13}C:^{12}C ratios of the sample and the standard, respectively [27]. Carbon isotope discrimination (CID), a factor related to isotope fractionation by the photosynthetic process relative to the source carbon was then estimated as $CID = (\delta_{air} - \delta_{plant})/(1 + \delta_{plant}/1000)$ where δ_{air} is the ^{13}C composition of atmospheric CO_2, which is assumed to be -8.0‰ [26]. Before calculating CID, oven-dried leaves of each plant were ground into a homogenous fine powder and 2-3 mg subsamples were weighed and placed into tin capsules (Elemental Microanalysis, UK) to be analyzed using a continuous flow Isotope Ratio Mass Spectrometer (Sercon Ltd., Cheshire, UK) at UC Davis Stable Isotope Facility (California, USA).

Genetic map construction

A set of 9832 SNPs were used to produce an Infinium HD iSelect BeadChip (Infinium). These SNPs were selected from either genomic re-sequencing or transcriptomic experiments. The gDNA from the INEDI RILs population obtained from the cross between XRQ and PSC8 lines (210 samples) were genotyped with the Infinium array. All genotyping experiments were performed by Integragen (IntegraGen SA, Genopole Campus 1 - Genavenir 8, 5 rue Henri Desbruères, 91000 Evry, France.) and the genotypic data were obtained with the Genome Studio software (Illumina) with automatic and manual calling. From the 9832 SNPs, 7094 were technically functional with more than 200 samples having a genotyping data. From this set of 7094 markers, 2576 were polymorphic between XRQ and PSC8 and 2164 did not show distortion of segregation in the population. We used CarthaGène v1.3 [34] to build the genetic maps. We added the genotypic data of markers from a consensus map [35] to the set of the 2164 SNPs to assign them to the appropriate LG to the *group 0.3 8* in CarthaGène. They were ordered using the *lkh 1 -1* function in CarthaGène for each group. The genetic map consisted of 2610 markers located on the 17 LG for a total genetic distance of 1863.1 cM and grouped on 999 different loci. All data will be available through the www.heliagene.org portal.

Statistical and QTL analysis

The data were first tested for normal distribution with the Kolmogorov-Smirnov test. These data were subjected to analysis of variance (ANOVA) and phenotypic correlation analysis (Pearson's correlation) using the software of statistical package PASW statistics 18 (IBM, New York, USA). Means were compared using a Student-Newman-Keuls (SNK) test ($P < 0.05$). The broad sense heritability (h^2) was then computed from the estimates of genetic ($\sigma^2 g$) and residual ($\sigma^2 e$) variances derived from the expected mean squares of the analyses of variance as $h^2 = \sigma^2 g/(\sigma^2 g+ \sigma^2 e/r)$, where r was the number of replicates.

QTL identification was performed using MCQTL, software for QTL analysis (http://carlit.toulouse.inra.fr/MCQTL/). The MCQTL software package can be used to perform QTL mapping in a multi-cross design. It allows the analysis of the usual populations derived from inbred lines [28]. MCQTL package is comprised of three software applications. The first component, TranslateData reads data from MAPMAKER [29] like files. The second component, ProbaPop computes QTL genotype probabilities given marker information at each chromosome location for each family and stores them in XML formatted files. The last component, Multipop builds the pooled model using the genotype probabilities, computes Fisher tests and estimates the model parameters [28]. The statistical significance of QTLs was

assessed using the MCQTL test, which is equal to –log(P-value (F-test)), as described in the MCQTL user guide.

Significant thresholds ($P < 0.05$) for QTL detection were calculated for each dataset using 1000 permutations [30] and a genome-wide error rate of 0.01 (Type I error). The corresponding type I error rate at the whole-genome level was calculated as a function of the overall number of markers in the map and the number of markers in each linkage group [31]. In our analysis, the threshold for the Fisher test (–log(P-value (F-test))) was 3.69 for both experiments. This threshold was an average of several thresholds of the traits at a significance level of 5% and was determined after 1000 permutations.

In each experiment, the QTL detection was also performed to identify QTL for the phenotypic response (called "response QTL"), calculated as the difference between two different water treatments (WW and WS). This allowed us to detect chromosome regions having quantitative effects on traits, depending on the environment [32-33].

Results

Genotypic variability and phenotypic correlation between water use efficiency (WUE) and carbon isotope discrimination (CID)

In general, a normal distribution was observed for WUE and CID traits across the two experiments and water treatments, except for WUE_{T2012} and CID in Exp. 2012 at WW conditions, the distributions deviate from normality according to the Kolmogorov-Smirnov test (Fig. 2 and 3). As normalizing data through transformation may misrepresent differences among individuals by pulling skewed tails toward the center of the distribution [30], all phenotypic analyses were performed on untransformed data.

Higher mean values for WUE for WS (2.31 to 3.06 $g.kg^{-1}$) than for WW (1.91 to 2.95 $g.kg^{-1}$) (Table S1 and S2) were observed in each experiment. In contrast, higher mean values for CID for WW than for WS were also observed in each experiment. In addition, a similar range of WUE and CID values was observed in both experiments for both WW and WS (for WUE in Exp. 2011 was represented by the WUE_{E2011}). In addition, significant genotypic effects were detected for all traits in Exp. 2011 (Table S1), and significant genotypic and SWC effects were detected for all traits in Exp. 2012 (Table 1).

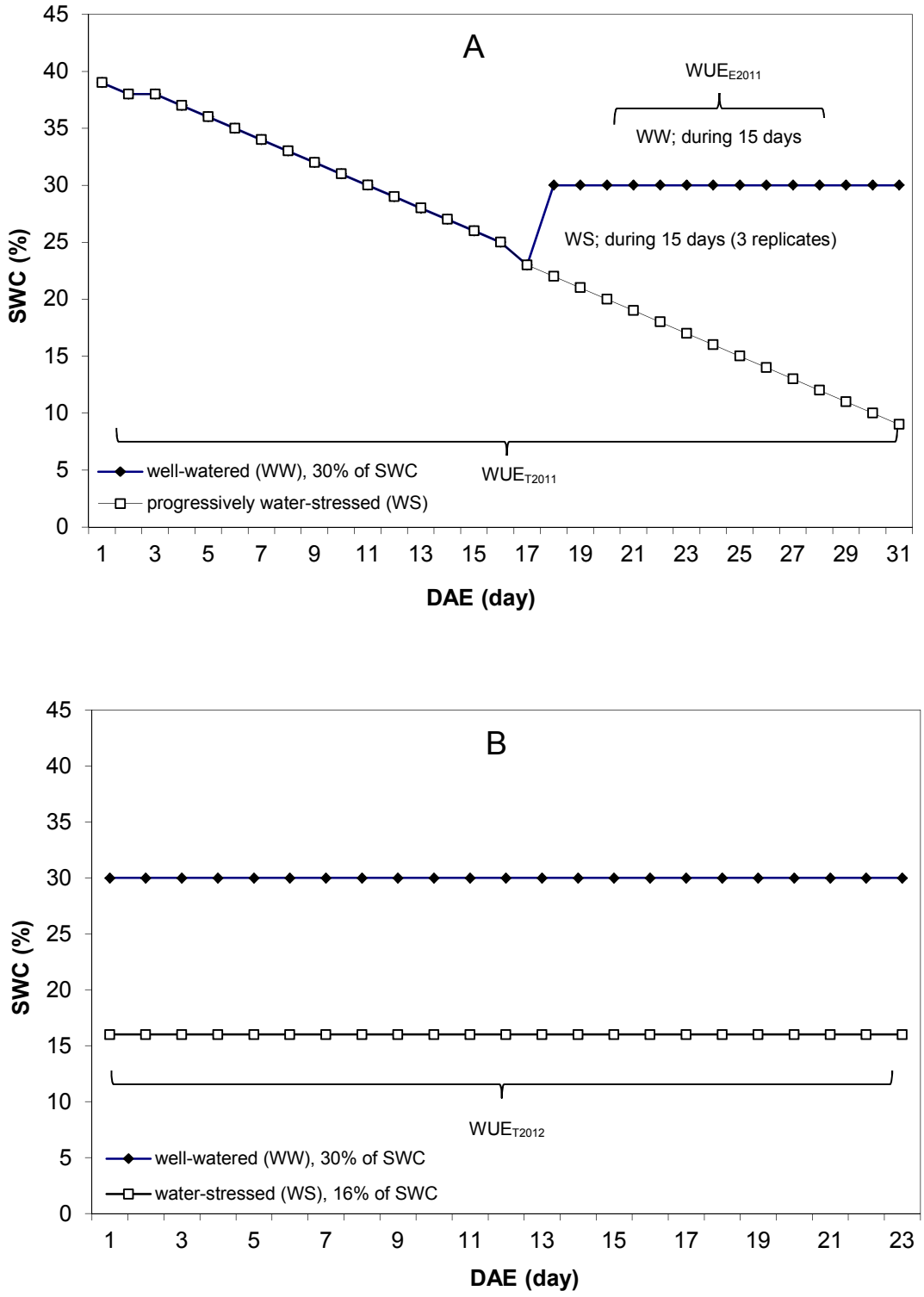

Figure 1. Principles of the water treatments used in this study. (A) In experiment 2011, three replicates (each of 150 plants) were subjected to progressive water-stress by water withholding from 1 to 31 DAE. In this experiment a control replicate (150 plants) was watered to maintain non-stressful conditions (SWC=30%). (B) In Experiment 2012, two replicates (each of 150 plants) were maintained at in stressful conditions SWC=16% from 1 to 23 DAE whereas two other replicates (each of 150 plants) were irrigated to maintain non-stressful conditions (SWC=30%). DAE: day after emergence.

The heritabilities of CID were usually higher than those of WUE in both experiments (CID with WUE_{T2011} or WUE_{T2012}), except that the heritability of WUE_{E2011} was higher than that of CID (Tables S1 and 1).

Significant negative correlations were observed between WUE and CID in both experiments (r_p = -0.197, $P < 0.05$; r_p = -0.409, $P < 0.001$; r_p = -0.565, $P < 0.001$ for the correlations of WUE_{T2011}, WUE_{E2011}, WUE_{T2012} with the CID, respectively; Fig. 4, Table S3). However, when we determined the correlation between WUE and CID for each treatment, we observed a positive correlation between the WUE_{T2011} and CID in Exp. 2011 for WS (Fig. 4 and Table S4). In addition, a significant phenotypic correlation was observed between Exp. 2011 and 2012 for both WUE and CID (Fig. 5)

QTL identified for water use efficiency (WUE)

In Exp. 2011, two QTL for WUE_{T2011} were detected for WW, four QTL for WUE_{E2011} were detected for WS and three "response QTL" for WUE (Table 2 and 4). For WW, the QTL were located on LG06 and LG11 with the highest likelihood odds ratio (LOD) value at 3 cM (QTL of *WUE11ww.11.1*) (Fig. S1). The marker for the QTL of *WUE11ww.11.1* was identified between the markers of HA005673_395 and HA006174_145 (Fig. 6). For WS, the QTL were located on chromosomes LG03 and LG16 (two QTL for each chromosome) with the highest LOD value at 6 cM, the QTL of *WUEe11ws.16.2*, and the marker of this QTL was HA017124_226. A "response QTL" for WUE (*WUE11diff.06.2*) was collocated with QTL of *WUE11ww.06.1*. In addition, two other "response QTL" were found on LG05 and LG06. The additive effects of the *WUE11ww.06.1* and *WUE11ww.11.1* were -0.14 and 0.11 while the additive effects of the *WUEe11ws.03.1, WUEe11ws.03.2, WUEe11ws.16.1,* and *WUEe11ws.16.2* were -0.13, 0.13, 0.38 and -0.44, respectively.

In Exp. 2012, two QTL for WUE_{T2012} were detected at WW and one QTL for WUE_{T2012} at WS (Table 3). For WW, the QTL were detected on chromosome LG13 and LG15 with the highest LOD value at 25 cM, the QTL of *WUE12ww.13.1*, and the markers for this QTL was restor (Fig. 6, Fig. S1). For WS, a QTL was detected on chromosome LG09 (QTL of *WUE12ws.09.1*) with the LOD value at 3 cM. The marker for the QTL of *WUE12ws.09.1* was identified between the markers of SSL053 and HA013641_506. In addition, a "response QTL" for WUE (*WUE12diff.13.1*) was co-located with the QTL of *WUE12ww.13.1* and *CID12ww.13.1*.. The additive effects of *WUE12ws.09.1, WUE12ww.13.1* and *WUE12ww.15.1* were 0.20, 0.04 and -0.06, respectively.

Table 1. Heritability (h^2) and mean square (MS) of analysis of variance (ANOVA) for water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) for 150 recombinant inbred lines (RILs), two stable soil water contents (SWC) and two replicates in Exp. 2012 (n = 600).

Trait	h^2	MS		
		Genotype	Soil water content	Genotype x soil water content
WUE_{T2012}	0.26	0.50***	28***	0.25^{ns}
CID	0.41	1.68***	1100***	0.53^{ns}
BM	0.36	0.51***	180***	0.29**
CWT_{23d}	0.36	40862***	31746440***	25565***

** Significant at $P < 0.01$, *** significant at $P < 0.001$.
^{ns} Not significant.

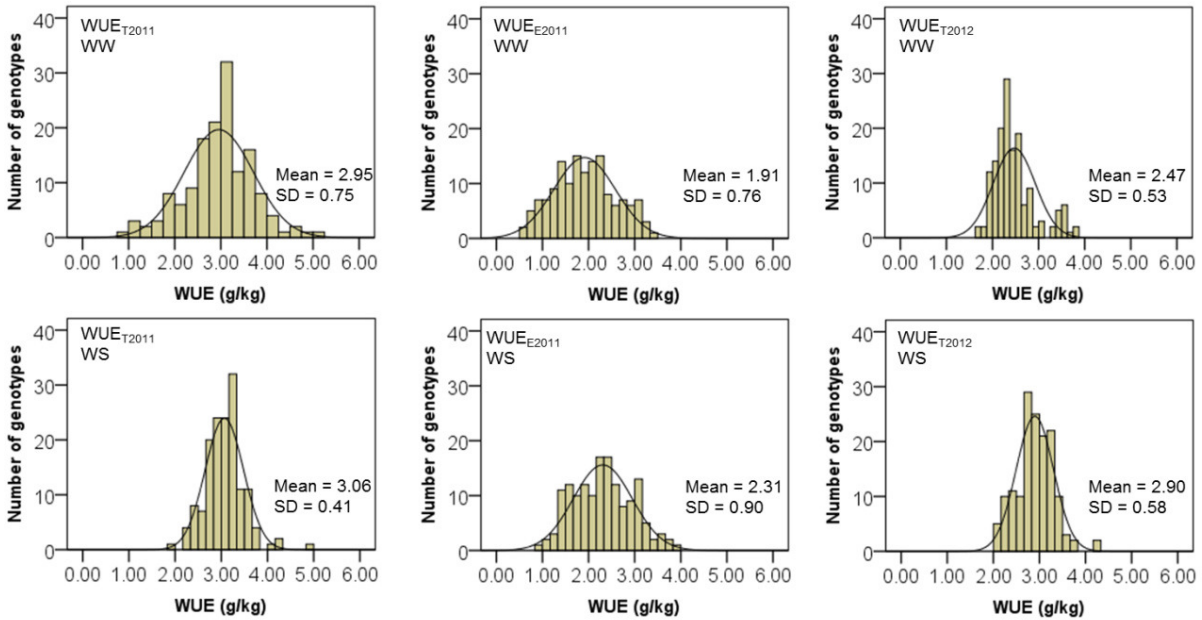

Figure 2. Frequency distribution for water use efficiency (WUE) in Exp. 2011 and 2012 of 150 recombinant inbred lines (RILs). WUE_{T2011}: total water use efficiency "total" in Exp. 2011; WUE_{E2011}: water use efficiency "estimation" in Exp. 2011; WUE_{T2012}: water use efficiency "total" in Exp. 2012. WW: well-watered; WS: water-stressed. For WUE_{T2011} and WUE_{E2011} at WW, data represent 150 RILs (n=150); for WUE_{T2011} and WUE_{E2011} at WS, data represent mean of three replicates of 150 RILs (n=150); for WUE_{T2012} at WW and WS, data represent mean of two replicates of 150 RILs (n=150). SD: standard deviation.

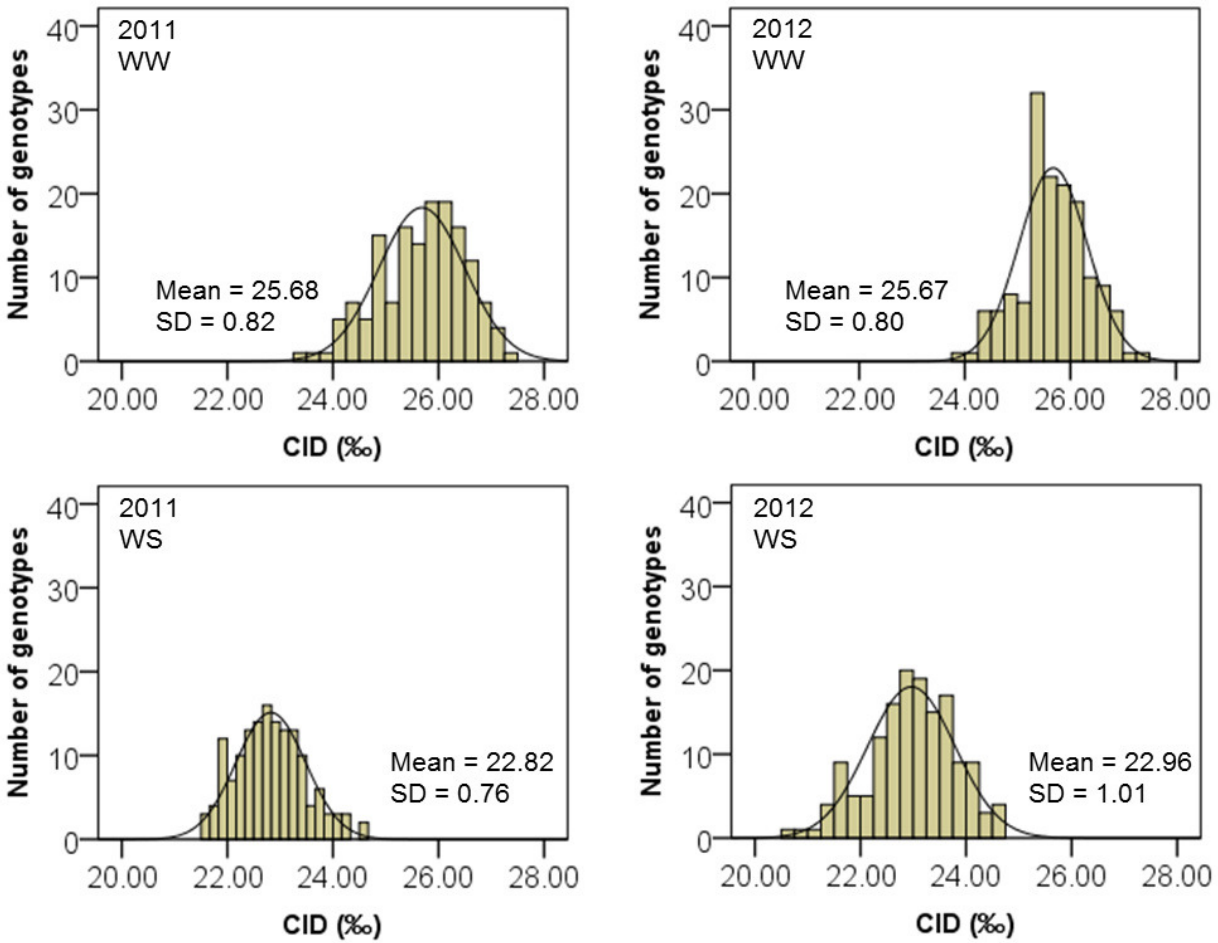

Figure 3. Frequency distribution for carbon isotope discrimination (CID) in Exp. 2011 and 2012 of 150 recombinant inbred lines (RILs). WW: well-watered; WS: water-stressed. For CID in Exp. 2011 at WW, data represent 150 RILs (n = 150); for CID in Exp. 2012 at WS, data represent mean of three replicates of 150 RILs (n = 150); for CID in 2012 at WW and WS, data represent mean of two replicates of 150 RILs (n = 150). SD: standard deviation.

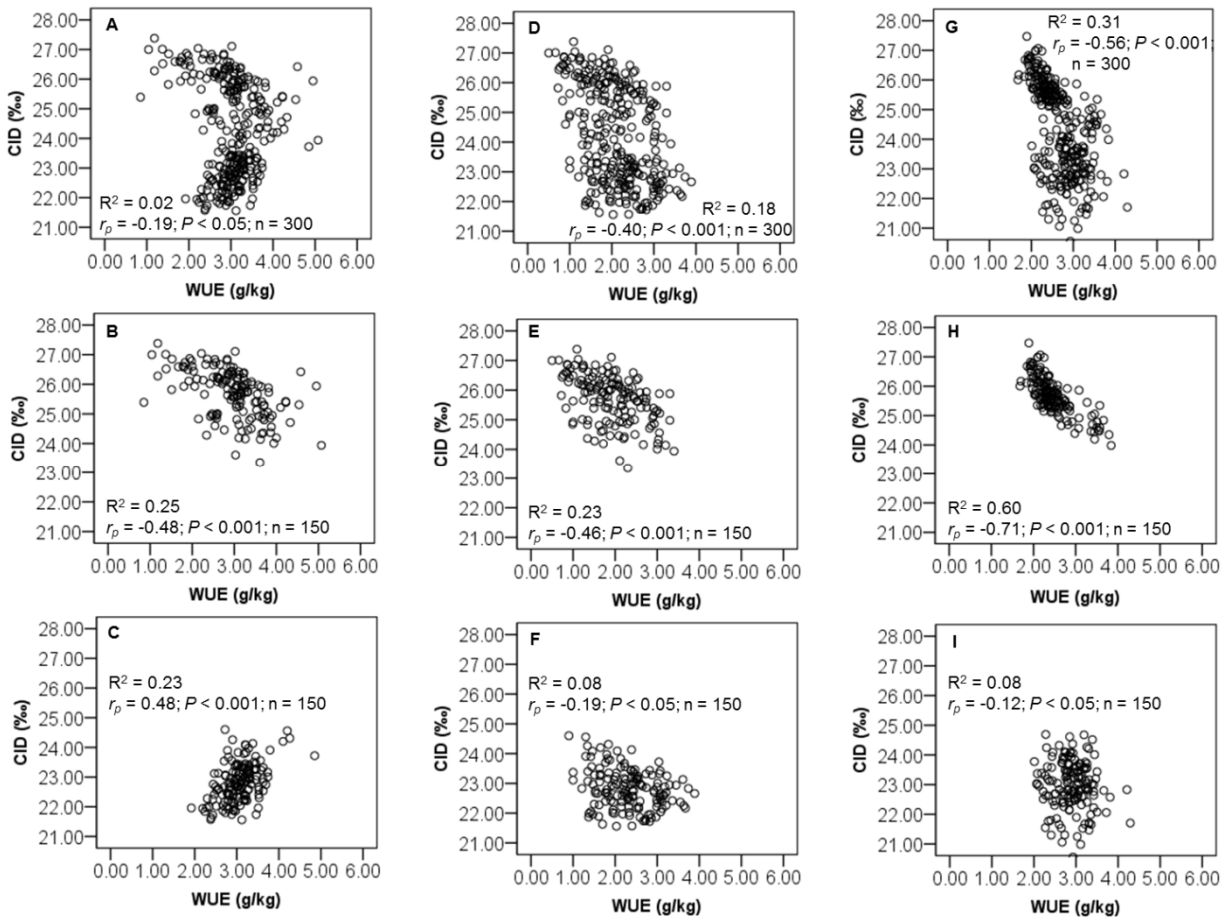

Figure 4. Relationship between water use efficiency (WUE) and carbon isotope discrimination (CID) of 150 recombinant inbred lines (RILs) in Exp. 2011 and Exp. 2012. Relationship between (A) WUE_{T2011} and CID in Exp. 2011, (B) WUE_{T2011} and CID at WW in Exp. 2011, (C) WUE_{T2011} and CID at WS in Exp. 2011, (D) WUE_{E2011} and CID in Exp. 2011, (E) WUE_{E2011} and CID at WW in Exp. 2011, (F) WUE_{E2011} and CID at WS in Exp. 2011, (G) WUE_{T2012} and CID in Exp. 2012, (H) WUE_{T2012} and CID at WW in Exp. 2012; (I) WUE_{T2012} and CID at WS in Exp. 2012. Phenotypic correlation (r_p) value is provided in each graph.

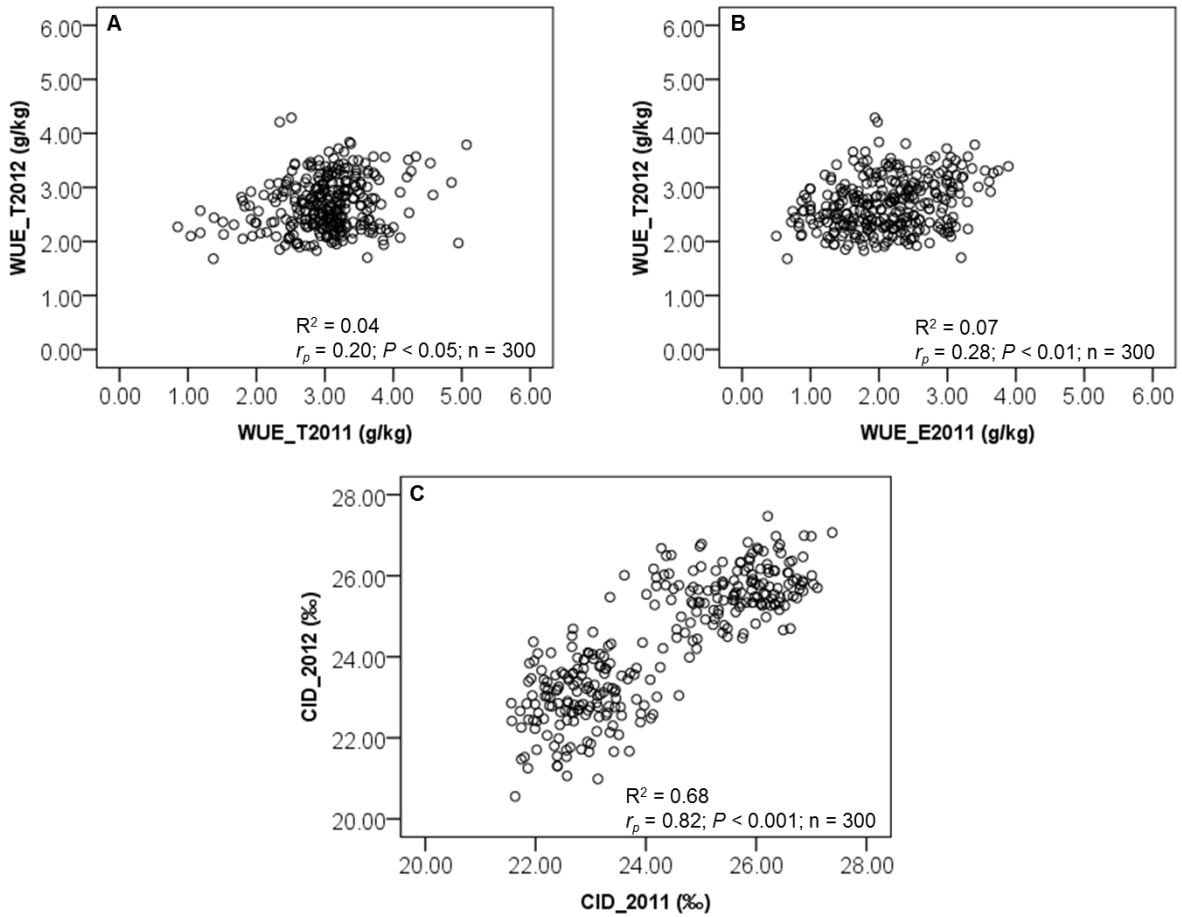

Figure 5. Relationship between (A, B) WUE and (B) CID values for 150 recombinant inbred lines (RILs) determined in two separate experiments (Exp. 2011 and 2012). For each trait and experiment, mean of well-watered (WW) and water-stressed (WS) plants were grouped together (n = 300). Phenotypic correlation (r_p) value is provided in each graph.

QTL identified for carbon isotope discrimination (CID)

In Exp. 2011, two QTL for CID were detected at WW and three QTL for CID were detected at WS (Table 2). For WW, the QTL were located on the same chromosomes of LG06 with the highest LOD value at 4.5 cM, QTL of *CID11ww.06.1*, and the marker of this QTL was ORS483 (Fig. 6, Fig. S2). For WS, the QTL were identified on chromosomes LG03, LG06 and LG13 with the highest LOD value at 5.5 cM, the QTL of *CID11ws.03.1*, and the marker of this QTL was HA013974_334. Besides, there was one "response QTL" detected for CID on chromosome LG02 (*CID11diff.02.1*) (Table 4). The additive effects were -0.15 and 0.12 (for QTL of *CID11ww.06.1* and *CID11ww.06.2*) while the additive effects were -0.13, -0.10, -0.13 (for the QTL of *CID11ws.03.1, CID11ws.06.1 and CID11ws.13.1*) (Table 2).

In Exp. 2012, two QTL for CID were detected at WW and one QTL for CID at WS (Table 3). For WW, the QTL were found on chromosomes LG13 and LG15 with the highest LOD value of 8.5 cM, the QTL of *CID12ww.13.1*, and the marker for this QTL was restor (Fig. 6, Fig. S2). For WS, a QTL was found on chromosome LG13 with an LOD value of 2.5 cM; the QTL of *CID12ws.13.1*, and the marker for this QTL was HACG0018_Contig_1_130. The additive effects for *CID12ww.13.1* and *CID12ww.15.1* were 0.20 and 0.07, respectively. The additive effects of the QTL of CID at WS (*CID12ws.13.1*) was 0.14.

QTL identified for related traits: biomass (BM) and cumulative water transpired (CWT)

In Exp. 2011, three significant QTL for BM, and one QTL for each of BM_E and CWT_{31d} at WS were identified (Table 2). These QTL were detected on chromosomes LG14, LG15, LG17, LG01 and LG11. There were only two "response QTL" detected for each of BM_E and CWT_{15d}. These QTL were detected on the same chromosome, LG06.

In Exp. 2012, seven QTL were identified for BM under both levels of SWC. For CWT_{23d}, five significant QTL were detected under both levels of SWC. Further, six "response QTL" for BM and CWT_{23d} were identified on chromosomes LG06, LG09, LG13 and LG15.

Table 2. Significant quantitative trait loci (QTL) detected for water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) under under well-watered and progressive water-stressed treatments in Exp. 2011.

Trait	Treatment	Chromosome	QTL name	QTL position (cM)	Inferior position (cM)	Superior position (cM)	R^{2a} (%)	R^2 global[b] (%)	Additive effect[c]
WUE_{T2011}	WW	LG06	*WUE11ww.06.1*	41.1	0	69.5	7	13	-0.14
	WW	LG11	*WUE11ww.11.1*	7.8	0	17.4	9	13	0.11
CID	WW	LG06	*CID11ww.06.1*	3	0.45	14.4	12	17	-0.15
	WW	LG06	*CID11ww.06.2*	47.3	23.6	60.1	8	17	0.12
WUE_{E2011}	WS	LG03	*WUEe11ws.03.1*	63.2	53.8	95.7	7	21	-0.13
	WS	LG03	*WUEe11ws.03.2*	97.7	63.9	124	5	21	0.13
	WS	LG16	*WUEe11ws.16.1*	94.1	92.7	96.1	11	21	0.38
	WS	LG16	*WUEe11ws.16.2*	97.1	96.1	99.3	15	21	-0.44
CID	WS	LG03	*CID11ws.03.1*	73.6	52	76.2	15	25	-0.13
	WS	LG06	*CID11ws.06.1*	11.3	0	16.6	9	25	-0.1
	WS	LG13	*CID11ws.13.1*	21.2	0	36.5	10	25	-0.13
BM	WS	LG14	*BM11ws.14.1*	42.4	0	108	5	17	0.01
	WS	LG15	*BM11ws.15.1*	76.3	0	98.9	7	17	0.02
	WS	LG17	*BM11ws.17.1*	76.1	0	112	6	17	-0.02
BM_E	WS	LG01	*BMe11ws.01.1*	67.8	46.3	74.9	9	9	0.02
CWT_{31d}	WS	LG11	*CWTe11ws.11.1*	9.1	0	20.5	7	7	-4.28

WW: well-watered, WS: progressive water-stressed.
[a] Phenotypic variance explained by QTL effect.
[b] Total of phenotypic variances explained by QTL effects.
[c] Additive effect estimated as one-half the difference in homozygotes carrying either allele of parents (XRQ or PSC8). Positive values indicate that XRQ allele increases the trait value, while negative values indicate that PSC8 allele increases the trait value.

Table 3. Significant quantitative trait loci (QTL) detected for water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) under well-watered and water-stressed treatments in Exp. 2012.

Trait	Treatment	Chromosome	QTL name	QTL position (cM)	Inferior position (cM)	Superior position (cM)	R^{2a} (%)	R^2 global[b] (%)	Additive effect[c]
WUE_{T2012}	WW	LG13	*WUE12ww.13.1*	26.2	25.14	26.95	42	45	0.2
	WW	LG15	*WUE12ww.15.1*	77.1	13.94	90.73	6	45	0.04
CID	WW	LG13	*CID12ww.13.1*	26.2	4.29	37.43	21	26	0.2
	WW	LG15	*CID12ww.15.1*	77.1	0	98.90	6	26	0.07
BM	WW	LG06	*BM12ww.06.1*	33.6	29.2	40.74	13	40	0.1
	WW	LG09	*BM12ww.09.1*	95.5	88.25	114.1	9	40	0.08
	WW	LG13	*BM12ww.13.1*	26.2	24.16	37.81	2	40	0.17
	WW	LG15	*BM12ww.15.1*	77.1	47.47	82.62	12	40	0.1
CWT_{23d}	WW	LG15	*CWT12ww.15.1*	79.1	40.28	87.03	7	7	26.07
WUE_{T2012}	WS	LG09	*WUE12ws.09.1*	55.5	33.28	83.25	9	9	-0.06
CID	WS	LG13	*CID12ws.13.1*	30.8	0	62.45	7	7	0.14
BM	WS	LG06	*BM12ws.06.1*	31.6	29.09	35.10	13	26	0.02
	WS	LG13	*BM12ws.13.1*	21.2	0	29.86	9	26	0.02
	WS	LG17	*BM12ws.17.1*	98.2	68.14	111	7	26	0.02
CWT_{23d}	WS	LG04	*CWT12ws.04.1*	6	0	21.48	10	30	-6.17
	WS	LG10	*CWT12ws.10.1*	33.6	0	112.5	4	30	3.23
	WS	LG15	*CWT12ws.15.1*	49.5	28.33	80.98	10	30	5.32
	WS	LG17	*CWT12ws.17.1*	89.8	74.7	92.29	12	30	-6.78

WW: well-watered (30% of SWC), WS: water-stressed (16% of SWC).

[a] Phenotypic variance explained by QTL effect.

[b] Total of phenotypic variances explained by QTL effects.

[c] Additive effect estimated as one-half the difference in homozygotes carrying either allele of parents (XRQ or PSC8). Positive values indicate that XRQ allele increases the trait value, while negative values indicate that PSC8 allele increases the trait value.

Table 4. Significant "response quantitative trait loci (QTL)" detected for water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) in Exp. 2011 and Exp. 2012.

Trait	Experiment	Chromosome	QTL name	QTL position (cM)	Inferior position (cM)	Superior position (cM)	R^{2a} (%)	R^2 global[b] (%)	Additive effect[c]
WUE_{T2011}	2011	LG05	*WUE11diff.05.1*	64	0	103	5	14	-0.7
	2011	LG06	*WUE11diff.06.1*	1.3	0	22.9	7	14	-0.1
	2011	LG06	*WUE11diff.06.2*	41.1	3	69.5	6	14	0.08
CID	2011	LG02	*CID11diff.02.1*	52.1	0	101	7	7	-0.1
BM_E	2011	LG06	*BMe11diff.06.1*	9.3	0	21.6	8	8	-0.1
CWT_{15d}	2011	LG06	*CWTe11diff.06.1*	15.9	0	22.4	9	9	-19
WUE_{T2012}	2012	LG13	*WUE12diff.13.1*	26.2	21.9	41.6	18	18	0.14
BM	2012	LG06	*BM12diff.06.1*	34.7	28.3	43	10	39	0.07
	2012	LG09	*BM12diff.09.1*	95.5	0.1	111	10	39	0.07
	2012	LG13	*BM12diff.13.1*	26.2	24.1	38.8	20	39	0.14
	2012	LG15	*BM12diff.15.1*	77.1	74.1	82.2	13	39	0.09
CWT_{23d}	2012	LG06	*CWT12diff.06.1*	35.5	24.9	43.7	10	16	25.5
	2012	LG15	*CWT12diff.15.1*	79.1	0	98.9	6	16	19.1

[a] Phenotypic variance explained by QTL effect

[b] Total of phenotypic variances explained by QTL effects

[c] Additive effect estimated as one-half the difference in homozygotes carrying either allele of parents (XRQ or PSC8). Positive values indicate that XRQ allele increases the trait value, while negative values indicate that PSC8 allele increases the trait value.

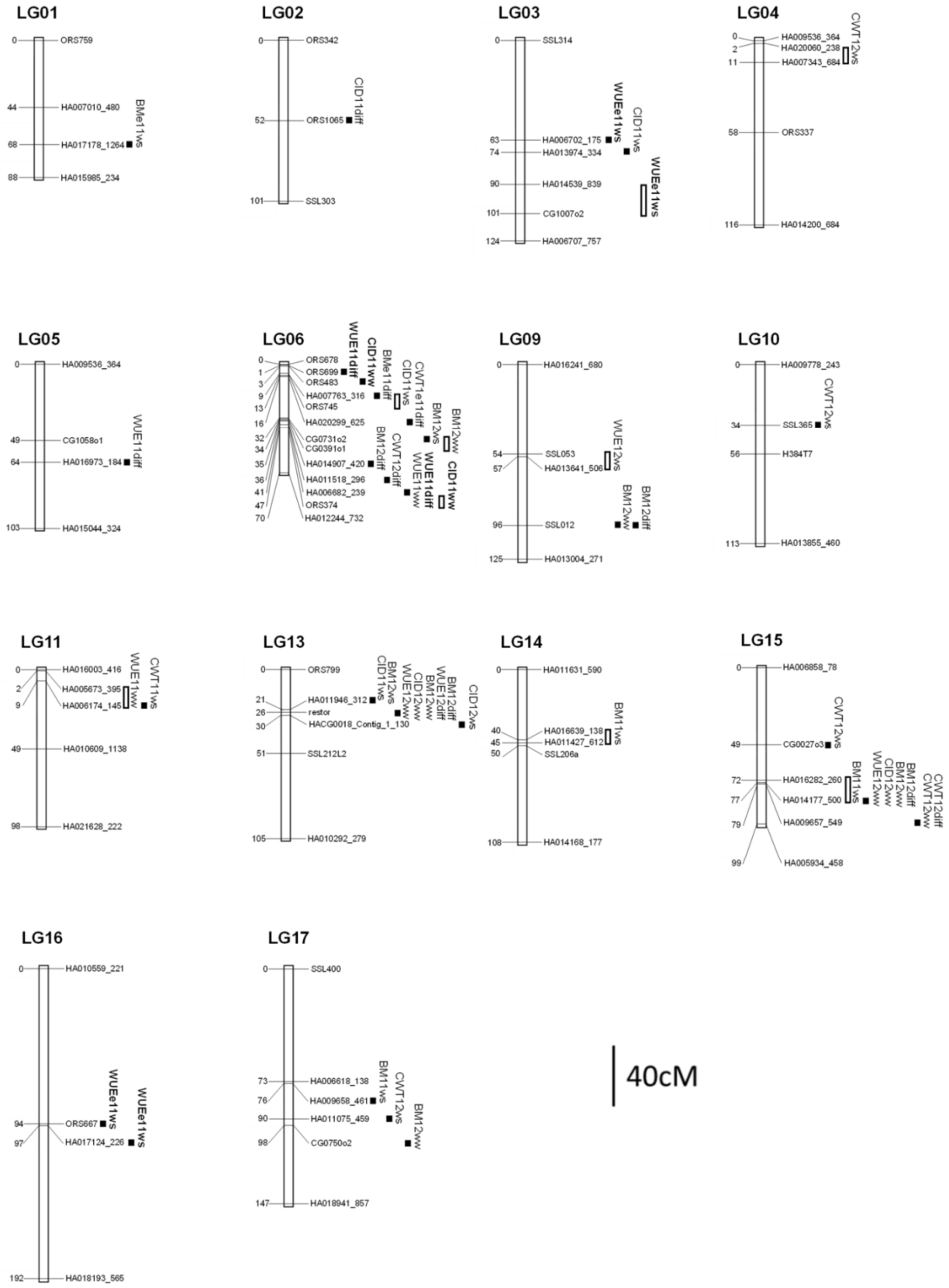

Figure 6. Genetic locations of QTL for water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) in the progressive stress experiment (2011) and the stable stress experiment (2012). Numbers on the left of linkage groups (LG) indicate the cumulative distance in centimorgan (cM) to the first marker at the top LG. Marker names and QTL are specified to the right of LG. The same QTLs which are found in a LG are shown in bold. Not all these chromosomes contain the complete markers (each chromosome has only been provided by the markers at the top, middle and bottom of LG as well as the markers for identified QTLs). QTL confidence intervals were estimated using the two-LOD confidence region

Discussion

Genetic variation and relationship between WUE and CID

In our experiments, increasing drought lead to an increase in WUE and a decrease in CID. This result was previously reported by Lauteri et al. [36] in sunflower and is well known in other crops, such as durum wheat (*Triticum turgidum* L.) [37], rice (*Oryza sativa* L.) [38] and eucalyptus (*Eucalyptus microtheca*) [39]. In addition, a similar range of values for WUE and CID was observed in the two experiments even though their water stress patterns differed. That was likely because the population had been constructed from parents that had specific responses in non-limited and limited water availability [40-41]. From the phenotypic data, XRQ exhibited low WUE while PSC8 exhibited high WUE (unpublished data).

CID is highly heritable trait and its heritability is usually higher rather than WUE [7,11]. Nevertheless, in the present study, both of CID and WUE were influenced by environmental variation because the heritability values were below 50% [24]. A previous study [42] has shown that heritabilities for CID, measured on detached sunflower leaves, were above 50% (74-96%), indicating that genetic variance for CID was dominant. However, this result was obtained for plants grown in optimal watering conditions. Consequently, CID appeared dependent on genetic and environmental control. This trait is genetically complex [43], and its expression in leaves and other plant tissues varies with the water supply. In drought conditions, Rebetzke et al. [24] reported that low soil water availability decreases stomatal conductance, which can reduce genetic variance and heritability of CID.

Our work demonstrated the clear relationship between WUE and CID in different water regimes. For each water regime and all genotypes, we observed negative correlations between WUE and CID. These results are in accordance with those of previous work in sunflower [36,42], and with those of numerous authors working on other crops [6,8,44-47]. In one case of progressive water stress, WUE_{T2011} and CID, were positively correlated. This was probably due to the high variability of the soil water content during the progressive drought establishment (SWC was gradually decreased). A similar result was reported on alfalfa genotypes [48]: WUE (mg of dry matter per g H_2O) was positively correlated with CID for plants subjected to progressive water stress during 7 days.

In the WW treatment, the high WUE was correlated with high BM and high CWT, while for the WS treatment the high WUE was still correlated to high BM but with low CWT. If increase in WUE is associated with reduced transpiration, such genotypes are often referred to as “conductance type”. On the other hand, if increase in WUE is correlated with increased photosynthesis, such genotypes can be categorized as “capacity types” [49-50]. Accordingly, the sunflower genotypes in our study can be categorized as an intermediate between

"conductance" and "capacity" type, unlike rice genotypes that have been categorized as "conductance type" [51]. In addition, our results were in agreement with several authors [39,52-53] who have suggested that plants that use water more efficiently by producing greater biomass for a given quantity of water transpired would grow more rapidly, resulting in a positive correlation between WUE and biomass production.

QTL identified for WUE and CID

Our study is the first to identify QTL for WUE and CID in sunflower subjected to drought. In Exp. 2011, significant regions affecting WUE were identified on four different chromosomes (LG03, LG06, LG11, LG16) in two water treatments and significant regions affecting CID were identified on three different chromosomes (LG03, LG06, LG13) for the same two water treatments. From these QTL, we observed a decrease and an increase of additive effects (XRQ), indicating that genes having both negative and positive effects had been involved in the difference in WUE and CID between the parental lines [54]. In Exp. 2012, the QTL for WUE were detected on three different chromosomes in two water treatments (LG09, LG13 and LG15) and the QTL for CID were identified on two different chromosomes in these two water treatments (LG13 and LG15). All these QTL increased the values of additive effects except the QTL of *WUE12ws.09.1,* indicating that XRQ allele increased the traits. These findings provide an explanation for the underlying genetic basis of the transgressive variation observed in the segregating population. This is in accordance with the argument proposed by Chapman et al. [55] and Vargas et al. [56], namely that a given QTL can have positive or negative additive effects, or none at all, depending on the drought scenario.

The WUE and CID were controlled by several QTL with small genetic additive (XRQ) effects, indicating that WUE and CID were genetically complex traits [2,57]. Reports evaluating genetic analysis for CID in other crops like soybean [58], cotton [59] and rice [54] have identified multiple QTL of smaller effect associated with the trait. However, in the present study, the QTL for WUE and CID explained 42% and 21% of the highest phenotypic variance (R^2). These R^2 values are higher than those found by previous authors for other crops, for example, rice [10,54], wheat [24] and barley [60-61].

Expression of QTL for WUE and CID across experiments and water treatments

The locations of QTL might be affected by growth stage [54] and/or environmental change [62-63]. In our results, the QTL for WUE_{T2011} and WUE_{E2011} were found on chromosomes LG03, LG06, LG11 and LG16 (under WW and WS), whereas the QTL for

WUE_{T2012} were found on chromosomes LG09, LG13 and LG15 (under WW and WS). These results showed that the expression of QTL for WUE differs with micro-environmental variations. This variation can be explained by the different water regimes in Exp. 2011 and Exp. 2012.

When the same mapping population is phenotyped in different environments, some QTL could be detected in one environment but not in others [63]. Collins et al. [64] noted that QTL can be categorized according to the stability of their effects across environmental conditions. A ‘‘constitutive’’ QTL is consistently detected across most environments, while an ‘‘adaptive’’ QTL is detected only in specific environmental conditions or increases in expression with the level of an environmental factor.

The QTL for CID in Exp. 2011 were detected on chromosomes LG03, LG06 and LG13 (WW and WS), whereas the QTL for CID in Exp. 2012 were detected on chromosomes LG13 and LG15 (under WW and WS). These results indicate that the expression of QTL for CID differs in the two experiments and different water regimes. Despite CID variation is influenced by stomatal conductance and photosynthetic capacity variations [7,37], several QTL of the different water regimes have been detected on the same chromosome [65-67]. This was the case in our study, where the three QTL for CID of the three different water regimes were detected on the same chromosome (LG13). Therefore, the QTL for CID in this study can be considered as a “constitutive” QTL. Additionally, the constitutive QTL for CID was consistent with the result of phenotypic correlation that genotypic ranking for this trait was consistently maintained in the two experiments.

Some QTL for WUE and CID and related traits were located on the same chromosome or on a similar QTL position (co-localization). The QTL for WUE_{T2012} for WW (*WUE12ww.13.1* and *WUE12ww.15.1*) had a similar QTL position (26.20 and 77.10 cM) as the QTL for CID for WW (*CID12ww.13.1, CID12ww.15.1*). The QTL for CID (*CID12ws.13.1*) for WS was associated with the QTL for WUE_{T2012} for WW (*WUE12ww.13.1*). This QTL was detected on chromosome LG13 (QTL position: 30.80 cM) near the QTL of *CID12ww.13.1*. The occurrence of QTL associated with different traits at the same locus may be explained by the fact that (i) the QTL are closely linked genetically or (ii) a single locus controls multiple traits and a gene may have pleiotropic effects [54].

We have observed a common genetic basis for WUE and CID in each experiment. Using the same mapping population under different water stress treatments helped us to characterize consistent genomic region (by QTL). Kiani et al. [68] indicated that QTL which was induced only by drought might be associated with mechanism(s) of sunflower drought response and they proposed that the QTL which can reduce trait difference between well-

watered and water-stressed conditions should have an effect on drought tolerance because of their contribution to trait stability. Our study in Exp. 2011 showed that the QTL for CID on chromosome LG06 were repeatable across two different water treatments (WW and WS). In Exp. 2012, the QTL for CID on chromosome LG13 have been repeatable across two different water treatments (WW and WS).

All these QTL which are common across different water treatments might be useful for marker-assisted selection (MAS). Identification of QTL influencing several traits could increase the efficiency of marker-assisted selection (MAS) and hasten genetic progress [69]. Ribaut et al. [70] noticed that in the design of the best-possible breeding strategy using MAS, additional traits and criteria have to be considered. For each trait of interest, some of the criteria are the number of QTL detected, the percentage of phenotypic variance that they explain, the total percentage of the genome that they represent, and their stability across different environments. Regarding these arguments, our study has shown that CID is the most interesting trait and should be useful for MAS, where three QTL overlapped on chromosome LG06 (CID for WW and WS in Exp. 2011), and three QTL across three different water treatments were co-localized on chromosome LG13 with phenotypic variance (R^2) ranges from 7 to 21%. Further, these QTL and other co-localized QTL on chromosomes LG06 and LG13 were identified in the near-centromeric region (inferior to superior position explained from 0 to 60.06 cM, and from 0 to 62.45 cM for LG06 and LG13, respectively), because those chromosomes are classified as a metacentric type [71-72].

Co-localization of QTL for WUE and CID with related traits

In this study, we also detected QTL for the related traits BM and CWT on the same chromosome of the QTL for WUE and/or CID (for WW and WS). These were observed in Exp. 2012, where two of four QTL for BM for WW (*BM12ww.13.1* and *BM12ww.15.1*) were detected on chromosomes LG13 and LG15, and co-located with the QTL for WUE_{T2012} and CID for WW (*WUE12ww.13.1, CID12ww.15.1*). For WS, the identifications of the QTL for the related traits showed a similar trend. The QTL of *BM12ws.13.1* (QTL position: 21.20 cM) was detected on chromosome LG13, as the QTL of *CID11ws.13.1, CID12ww.13.1, CID12ws.13.1* and *WUE12ww.13.1* have been identified. These indicated the possibility of genetic association of WUE and CID with the accumulation of biomass. Consistent with this, Kiani et al. [68] identified a QTL for total dry matter in water-stressed conditions on chromosome LG13 using another population of sunflower. Interestingly, this QTL overlapped with osmotic adjustment, grain yield, and plant height. Thereby the common genetic basis for WUE, CID, productivity and osmotic adjustment will lead to an improved understanding of

drought tolerance genes. In addition, evidence of overlapping QTL of productivity and osmotic adjustment have been observed by several authors [73-75]. However, further study is obviously required to determine the genetic control of osmotic adjustment or hydraulic conductance and their inter-relationships with WUE and CID.

For CWT, the QTL of *CWT12ww.15.1* was detected on chromosome LG15 with the QTL position at 79.10 cM near the marker at position of 77.10 cM where the QTL of *WUE12ww.15.1* and *CID12ww.15.1* have been identified. Not far from these positions, a QTL of *CWT12ws.15.1* was also detected (QTL position: 49.5 cM). These indicated out that the cumulative water transpired in WW and WS is genetically and closely related with WUE and CID in non-limited water availability. In addition, the maintenance of biomass accumulation under stable water stress should be considered as an efficiency process between transpiration, biomass accumulation and its partitioning between non-drought and drought conditions [64]. Therefore, the increase in WUE (i.e. the amount of biomass produced per unit of transpired water) might seem to be ideal candidate mechanism for drought-prone environments.

Identifying the "response QTL" for WUE and CID

In our work, we calculated the "response QTL" to provide new insight into the genetic architecture of WUE and CID, which, unlike a "common" phenotypic trait, is rarely considered in QTL analysis. Water use traits and their response are of primary importance to plant growth and survival. Although we have a growing understanding of the genetic and molecular drivers of water use traits and WUE as well as CID, response QTL of those traits has received relatively little attention.

We detected three QTL of "response QTL" for WUE on chromosomes LG06 and LG13. From these two chromosomes we have also identified the QTL for WUE_{T2011} and WUE_{T2012} for WW, indicating, at least under the conditions imposed in these experiments, that response QTL was controlled by loci that determine the main trait value under a specific treatment. This was in agreement with Kliebenstein et al. [76-77] who evaluated the response QTL between control and methyl jasmonate (MeJa)-treated plants of *Arabidopsis thaliana*. They reported that significant QTL that influenced response between control and MeJa-treated plants also affected the main trait value in at least one of the two environments, which was called the "allelic sensitivity" model.

In contrast, an independent response QTL, was also observed for several traits, for example the response QTL for WUE_{T2011} on chromosome LG15 (*WUE11diff.05.1*), CID on chromosome LG02 (*CIDdiff11.02.1*), and CWT_{23d} on chromosome LG06 (*CWT12diff.06.1*). This observation was not consistent with Kliebenstein et al. [77], however, it was in

agreement with an argument of Schlichting and Pigliucci [78] who suggested the "gene regulation" model must exist, and is not always controlled by loci that are expressed within at least one of the two environments.

As for the prospects for these aspects, characterization of the genes underlying QTL that control the differential WUE and CID regulation might generate a detailed understanding of the molecular and biochemical basis for water use traits in sunflower and how this alters phenotypic response in more complex environments.

Importance of high WUE or low CID for sunflower breeding: use of the identified markers for MAS

This is the first genetic quantitative analysis and QTL mapping for WUE and CID in sunflower. We investigated two drought scenarios and evaluated genetic variation of sunflower lines to identify genetic control and physiological processes that could explain genotypic differences in the response to drought stress. The present study proved that, in sunflower, selection for CID can be considered in initial screening to improve WUE. However, this merits further investigation in other populations.

Many QTL (particularly for CID) have been reported in the literature. However, very few with large effects have been adequately exploited in crop breeding programs. The majority of the favorable alleles for identified QTL are to be found in journals on library shelves rather than in crop cultivars improved by introgression or selection of these favorable QTL alleles [79]. Nevertheless, Condon et al. [80] reported the release of a new high-yielding wheat variety in droughted environments after a breeding process in which selection for low CID in non-droughted plants led to high WUE.

In conclusion, our results emphasize that the near-centromeric region of chromosomes LG06 and LG13 are a "reliable" region for MAS due to the co-localization of the QTL for CID with several QTL for WUE, BM and CWT. Indeed, the best strategy for using molecular markers should combine selection for QTL involved in the expression of CID.

This paper complements the study of Vincourt et al. [25] and Rengel et al. [81] that exploited the INEDI RIL population in analyzing genetic variation of agronomic and physiological traits, making it possible to establish strategies for a sunflower breeding program and provide a basis for identification of the molecular components of a genotype x environment interaction.

Acknowledgments

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Supporting Information

Figure S1 Genetic maps and LOD positions showing the locations of QTLs controlling WUE identified by MCQTL.

(DOCX)

Figure S2 Genetic maps and LOD positions showing the locations of QTLs controlling CID identified by MCQTL.

(DOCX)

Table S1 Genotypic variation of water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) for 150 recombinant inbred lines (RILs) under well-watered (WW) and progressively water-stressed (WS) treatments in Exp. 2011.

(DOCX)

Table S2. Genotypic variation of water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) for 150 recombinant inbred lines (RILs) for well-watered (WW) and water-stressed (WS) in Exp. 2012.

(DOCX)

Table S3. Phenotypic correlations (r_p) between water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) of 150 recombinant inbred lines (RILs) in Exp. 2011 and Exp. 2012.

(DOCX)

Table S4. Phenotypic correlations (r_p) among water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) of 150 recombinant inbred lines (RILs) under well-watered (WW) and progressive water-stressed (WS) treatments in Exp. 2011.

(DOCX)

Table S5. Phenotypic correlations (r_p) among water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) of

150 recombinant inbred lines (RILs) under well-watered (WW) and water-stressed (WS) treatments in Exp. 2012.
(DOCX)

Authors Contributions

Conceived and designed the experiments: ALA, PG, TL. Performed the experiments: ALA, PG, ON. Analyzed the data: ALA, PG, NL. Contributed reagents/materials/analysis tools: ALA, PG, NL, SM. Coordinated research: PG, TL. Wrote the paper: ALA, PG.

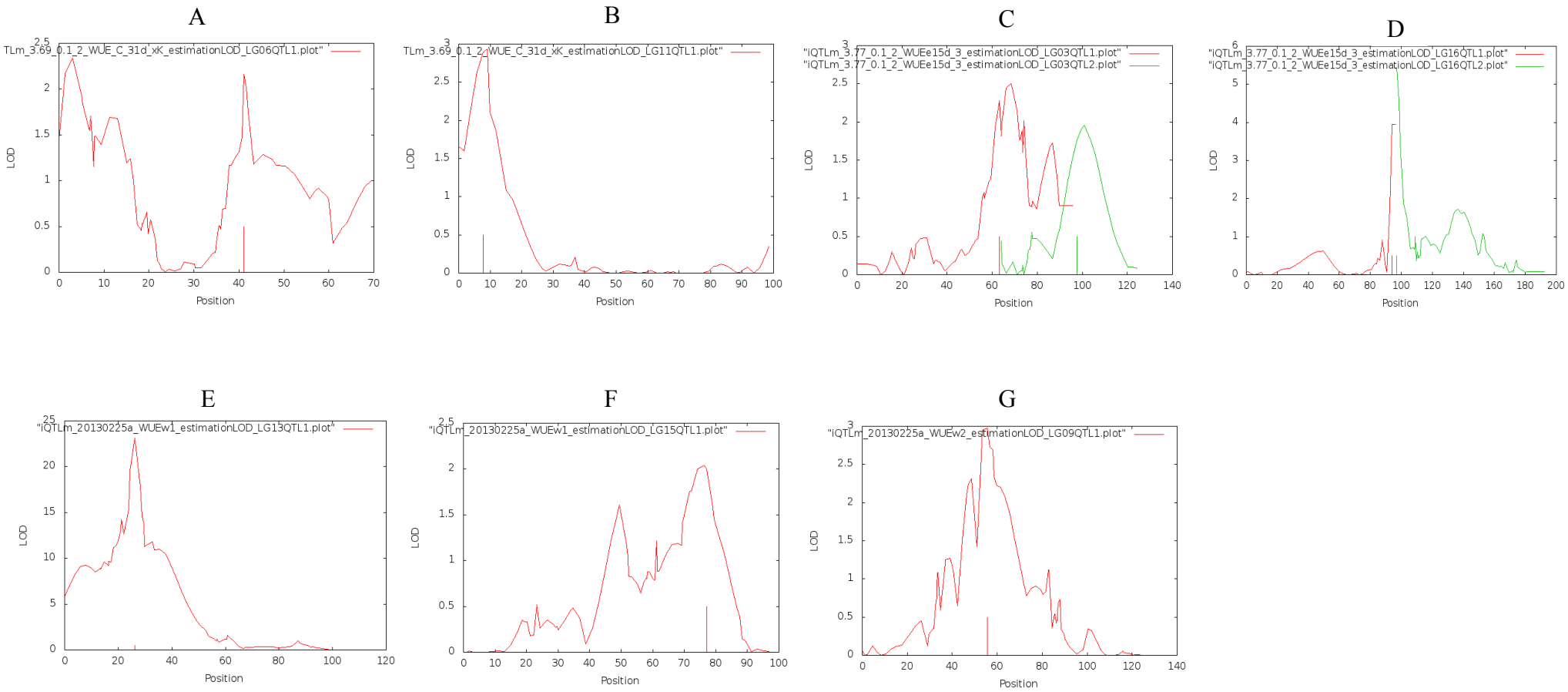

Figure S1. Genetic maps and LOD positions showing the locations of QTLs controlling WUE identified by MCQTL. These figures present the QTLs for WUE_{T2011} at WW on LG06 (A), WUE_{T2011} at WW on LG11 (B), WUE_{E2011} at WS on LG03 (C), WUE_{E2011} at WS on LG16 (D), WUE_{T2012} at WW on LG13 (E), WUE_{T2012} at WW on LG15 (F) and WUE_{T2012} at WS on LG09 (G). Notifications (iQTLm) on each map in these figures were only used for the authors.

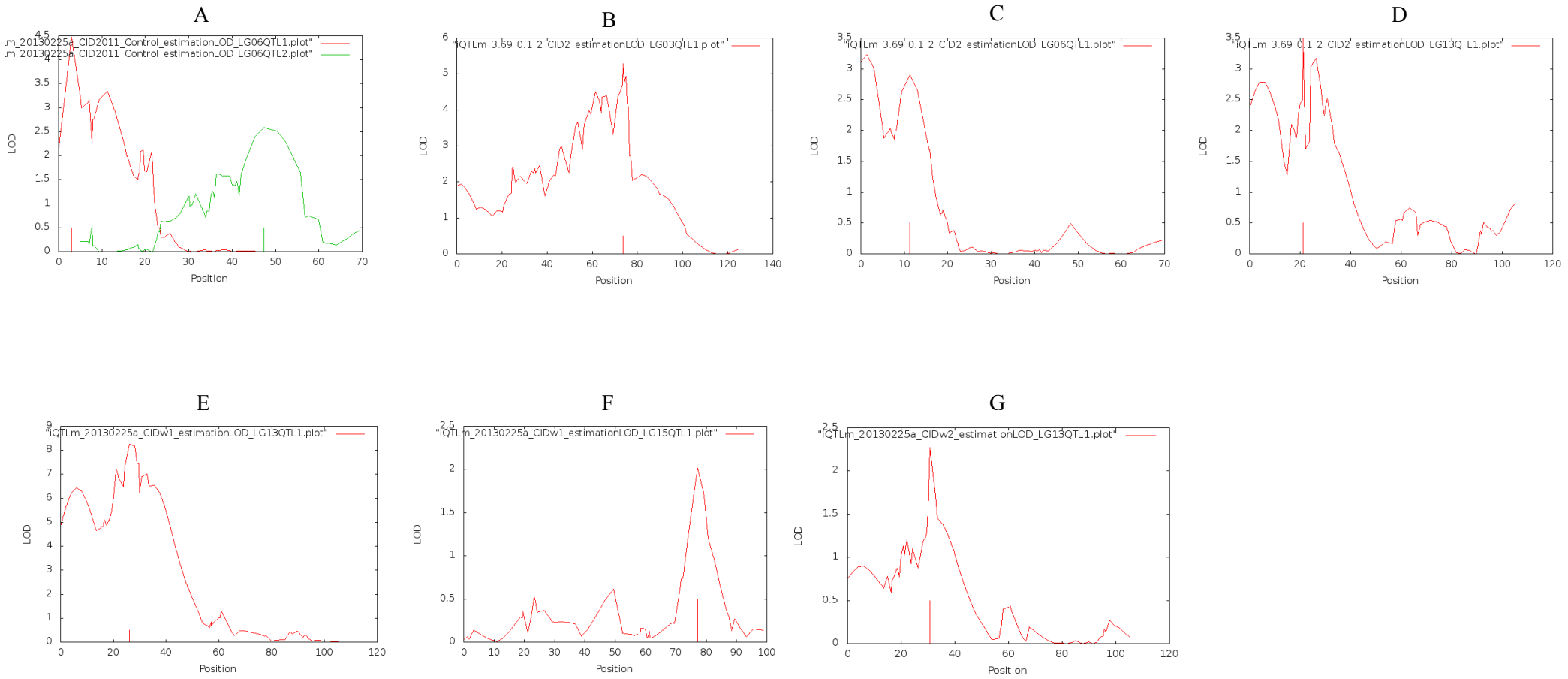

Figure S2. Genetic maps and LOD positions showing the locations of QTLs controlling CID identified by MCQTL. These figures present the QTLs for CID 2011 at WW on LG06 (A), CID 2011 at WS on LG03 (B), CID 2011 at WS on LG06 (C), CID 2011 at WS on LG13 (D) CID 2012 at WW on LG13 (E), CID 2012 at WW on LG15 (F) and CID 2012 at WW on LG13. Notifications (iQTLm) on each map in these figures were only used for the authors.

Table S1. Genotypic variation of water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) for 150 recombinant inbred lines (RILs) under well-watered (WW) and progressively water-stressed (WS) treatments in Exp. 2011.

Trait	**WW**						
	Minimum	**Maximum**	**Mean**	**Std.deviation**	**Variance**		
WUE_{T2011} ($g.kg^{-1}$)	0.85	5.07	2.95	0.75	0.56		
WUE_{E2011} ($g.kg^{-1}$)	0.5	3.4	1.91	0.76	0.57		
BM (g)	0.57	5.18	2.5	0.91	0.84		
BM_E (g)	0.2	3.87	2.49	0.93	0.33		
CWT_{31d} (ml)	522	1270	831	175	30759		
CWT_{15d} (ml)	294	903	585	108	11675		
CID (‰)	23.35	27.38	25.68	0.82	0.67		
	WS						
	Minimum	**Maximum**	**Mean**	**Std.deviation**	**Variance**	h^2	**MSg**
WUE_{T2011} ($g.kg^{-1}$)	1.92	4.85	3.06	0.41	0.17	0.17	0.17***
WUE_{E2011} ($g.kg^{-1}$)	0.9	3.89	2.31	0.90	0.80	0.46	1.87***
BM (g)	0.78	1.51	1.18	0.12	0.01	0.20	0.04***
BM_E (g)	0.11	0.94	0.54	0.16	0.24	0.78	0.07***
CWT_{31d} (ml)	271	439	389	28.20	795	0.02	2967***
CWT_{15d} (ml)	98	400	235	47.11	2219	0.15	3965***
CID (‰)	21.09	25.78	22.82	0.76	0.57	0.44	1.31***

*** Significant at $P < 0.001$.
h^2: heritability, MSg: mean square of genotype.
For WW, data represent 150 RILs. For WS, data represent mean of three replicates of 150 RILs (n=150) whereas MSg and h^2 were calculated from three replicates (n=450).

Table S2. Genotypic variation of water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) for 150 recombinant inbred lines (RILs) for well-watered (WW) and water-stressed (WS) in Exp. 2012.

Trait	**WW**					
	N	**Minimum**	**Maximum**	**Mean**	**Std.deviation**	**Variance**
WUE_{T2012} ($g.kg^{-1}$)	300	1.3	4.46	2.47	0.53	0.28
CID (‰)	300	23.69	27.8	25.67	0.80	0.64
BM (g)	300	0.48	4.67	1.66	0.76	0.57
CWT_{23d} (ml)	300	257	1248	659	216	46688
	WS					
	N	**Minimum**	**Maximum**	**Mean**	**Std.deviation**	**Variance**
WUE_{T2012} ($g.kg^{-1}$)	300	1.52	4.68	2.90	0.58	0.33
CID (‰)	300	20.01	25.37	22.96	1.01	1.02
BM (g)	300	0.25	1.93	0.57	0.17	0.03
CWT_{23d} (ml)	300	11	424	198	49.00	2401

WW = 30% of soil water content (SWC), WS = 16% of SWC.
N: number of plants.

Table S3. Phenotypic correlations (r_p) between water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) of 150 recombinant inbred lines (RILs) in Exp. 2011 and Exp. 2012.

Trait	**Experiment 2011**		
	WUE_{T2011}	**CID**	**BM**
CID	-0.197*		
BM	0.409***	0.457***	
CWT_{31d}	0.112^{ns}	0.580***	0.913***
	WUE_{E2011}	**CID**	**BM_E**
CID	-0.409***		
BM_E	0.420***	0.374***	
CWT_{15d}	-0.204***	0.739***	0.748***
	Experiment 2012		
	WUE_{T2012}	**CID**	**BM**
CID	-0.565***		
BM	-0.005^{ns}	0.550***	
CWT_{23d}	-0.314***	0.707***	0.936***

* Significant at $P < 0.05$, *** Significant at $P < 0.001$.
^{ns} Not significant.
For each experiment, mean of well-watered (WW) and water-stressed (WS) plants were grouped together (n = 300).

Table S4. Phenotypic correlations (r_p) among water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) of 150 recombinant inbred lines (RILs) under well-watered (WW) and progressive water-stressed (WS) treatments in Exp. 2011.

Trait	**WW**		
	WUE_{T2011}	**CID**	**BM**
CID	-0.488***		
BM	0.888***	0.700***	
CWT_{31d}	0.518***	0.638***	0.835***
	WUE_{E2011}	**CID**	**BM_E**
CID	-0.466***		
BM_E	0.941***	-0.575***	
CWT_{15d}	0.416***	-0.598***	0.660***
	WS		
	WUE_{T2011}	**CID**	**BM**
CID	0.480***		
BM	0.802***	0.135^{ns}	
CWT_{31d}	-0.478***	-0.564***	0.065^{ns}
	WUE_{E2011}	**CID**	**BM_E**
CID	-0.192*		
BM_E	0.837***	0.018^{ns}	
CWT_{15d}	-0.484***	0.386***	0.034^{ns}

* Significant at $P < 0.05$, ** Significant at $P < 0.01$, *** Significant at $P < 0.001$.
^{ns} Not significant.
For WW, values represent 150 RILs. For WS, values represent mean of three replicates of 150 RILs (n = 150).

Table S5. Phenotypic correlations (r_p) among water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) of 150 recombinant inbred lines (RILs) under well-watered (WW) and water-stressed (WS) treatments in Exp. 2012.

Trait	**WW**		
	WUE_{T2012}	**CID**	**BM**
CID	-0.718***		
BM	0.734***	-0.717***	
CWT_{23d}	0.322***	-0.522***	0.863***
	WS		
	WUE_{T2012}	**CID**	**BM**
CID	-0.121*		
BM	0.546***	0.110^{ns}	
CWT_{23d}	-0.226***	-0.022^{ns}	0.661***

* Significant at $P < 0.05$, *** Significant at $P < 0.001$.
^{ns} Not significant.
WW = 30% of soil water content (SWC), WS = 16% of SWC.
For each treatment, values represent mean of two replicates of 150 RILs (n = 150).

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From the results of the first article (Section 4.1), the genetic control of WUE and CID was able to be identified through QTL analysis. From that study, QTL associated with WUE and CID was co-localized across two different experiments and drought scenarios (drought scenario 1 and 2). Nevertheless, from drought scenario 1 (experiment in 2011), there were several plant-water relation traits which could reveal the variation of WUE and CID. Therefore, to achieve this goal, it is required to identify the genetic control of plant-water relation traits, i.e. transpiration control, water extraction capacity and dehydration tolerance in that drought scenario. The variation of the plant-water relation traits allows revealing the variation of WUE and CID.

4.2 Genetic analysis of transpiration control, water extraction capacity, and osmotic adjustment in sunflower (*Helianthus annuus* L.) under drought

Afifuddin Latif Adiredjo[1,2], Pierre Casadebaig[3], Nicolas Langlade[5,6], Thierry Lamaze[4,*], Philippe Grieu[1,*]

[1]Université de Toulouse, INP-ENSAT, UMR1248 AGIR (INPT-INRA), Castanet-Tolosan, France; [2]Brawijaya University, Faculty of Agriculture, Department of Agronomy, Plant Breeding Laboratory, Malang, Indonesia; [3]UMR1248 AGIR (INPT-INRA), Castanet-Tolosan, France; [4]Université de Toulouse, UPS-Toulouse III, UMR5126 CESBIO, Toulouse Cedex 9, France; [5]INRA, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, Castanet-Tolosan, France; [6]CNRS, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR2594, Castanet-Tolosan, France, *PhD supervisors of the first author

Submitted to Theoritical and Applied Genetics

Author for correspondence :
Philippe Grieu
Tel.: +33(0)534323878
Email: grieu@ensat.fr

Summary

- Stomatal control of transpiration (FTSWt), soil water extraction capacity (TTSW) and osmotic adjustment (OA), as dehydration tolerance criteria, are all involved in the main strategies whereby plants cope with water stress. Here we investigated the genetic control of these traits.
- Using recombinant inbred lines (RILs) of sunflower, we conducted a progressive water deficit experiment and analyzed the variation of FTSWt, TTSW and OA and their interaction. Quantitative trait loci (QTL) mapping was then performed to determine the loci involved and to identify the genetic control.
- This paper is the first report for QTL mapping of FTSWt, and is the second report for QTL mapping of TTSW in crops.
- The traits were significantly different between genotypes and showed a significant inter-relationship for FTSWt and TTSW. This interaction was consistent with the analysis of QTL co-localization. A QTL of FTSWt co-localized with QTL of TTSW on chromosome LG14.
- The genetic control between FTSWt and OA, as well as between TTSW and OA was independent, as no co-localization of QTL was observed.

Keywords: sunflower, drought, genetic, fraction of transpirable soil water (FTSW), total transpirable soil water (TTSW), osmotic adjustment (OA).

Introduction

Soil water availability is the major factor limiting plant productivity (Jones, 2013). The expansion of cropping into water-limited environments lends urgency to developing crop genotypes that use water more efficiently (Sinclair, 2011). The water status of a plant is directly related to the difference between the flow of water into the roots and that being transpired by the leaves at any given time (Comstock, 2002). Drought experienced by the plant is defined, at every moment, by water conditions in the plant, soil and air (Blum, 1998). Different plants subjected to the same water stress do not respond to this stress in the same way. A wide range of mechanisms has been summarized by Tardieu *et al.* (2007) and, in addition, a significant genotypic variability of these mechanisms has been studied in many crops (Turner *et al.,* 1986; Sinclair *et al.,* 1998; Poormohammad Kiani *et al*., 2007a).

First of all, the plant can reduce transpiration by closing its stomata, reducing stomatal conductance which determines gas exchange (CO_2 and H_2O). Ritchie (1981) proposed that there might be a water stress response function that is common to most soils. He found that plants initiate a linear decline in transpiration rate once the fraction of transpirable soil water (FTSW) has decreased to about one-third of the total transpirable soil water (TTSW). After reaching that threshold (FTSWt), transpiration rate decreased linearly with further soil drying (Devi e*t al*., 2009). Later, variability of FTSWt has been reported in a wide range of crop species and environmental conditions (Sinclair & Ludlow, 1986; Sadras & Milroy, 1996; Lacape *et al*., 1998; Lebon *et al*., 2003; Masinde *et al*., 2006; Casadebaig *et al*., 2008; Kholova *et al*., 2010; Gholipoor *et al*., 2013).

On a scale of several days, plants adjust their transpiration by decreasing leaf area and growth rate, leading to reduction of shoot and root growth. It has been shown that deep rooting results in an increased capability to extract soil water from depth and improve plant water status, and may increase yield under drought (Yeo, 2007). There appears to be convincing evidence that deeper and thicker roots should contribute to drought tolerance in at least some environments (Price *et al*., 2002). Some authors have proposed the measurement of TTSW to determine the capability of plants to extract water from the soil (Sinclair & Ludlow, 1986; Casadebaig *et al*., 2008; Marguerit *et al*., 2012). The optimization of water absorption is related to complex morphological characteristics of roots, in terms of mass, volume, and branching depth (Ramanjulu & Bartels, 2002). Studies on water extraction by different genotypes involving TTSW have rarely been reported because two types of reasons limit the use of many root criteria by breeders (Turner *et al.,* 2001; Sinclair, 2011): (i) the impracticality of field screening for this feature on a large scale and the difficulty of correlating field observations with those made in pots, (ii) the lack of a precise understanding

of the exact role of the roots in water-limited conditions is another limiting factor when devising a screening system (Passioura, 1994). Moreover, water extraction is closely related to the hydraulic conductivity of plant tissue, including roots (Sinclair, 2005). In this paper, we provide new insight into the genetic control of TTSW, which is rarely studied in crops (Marguerit *et al*., 2012).

Another mechanism allowing crops to tolerate water stress is osmotic adjustment (OA) (Morgan, 1983; Turner *et al*., 1986; Santamaria *et al*., 1990; Chimenti & Hall, 1994; Patakas *et al*., 2002). OA is an adaptive process which can reduce some of the harmful effects of water deficits. The main role of osmotic adjustment is to assist in the maintenance of turgor and volume as organs lose water during desiccation (Turner & Jones, 1980; Teulat *et al*., 1998), i.e. to provide dehydration tolerance (Saranga *et al*., 2004). This trait has a positive direct or indirect effect on plant productivity under drought stress (Ludlow & Muchow, 1990; Chimenti *et al*., 1996).

In this study we used sunflower recombinant inbred lines (RILs) as a crop model for quantitative trait loci (QTL) mapping. This crop is often reported as being drought-tolerant (Merrien *et al*., 1981; Connor & Hall, 1997), but this tolerance varies with the cultivar. That is why, in this paper, we study the plant-water relation traits, i.e. transpiration control (FTSWt), water extraction capacity (TTSW) and dehydration tolerance (OA) of sunflower under drought by analyzing its variability and mapping the genomic regions that are responsible for those traits through QTL analysis. QTL analysis provides the opportunity to compare whether different traits have a common genetic basis (Tanksley, 1993; Lynch & Walsh, 1998). Besides, an understanding of the sources of genetic variation and physiological mechanisms involved facilitates the development of an appropriate strategy to breed drought-tolerant cultivars (Sinclair, 2011)

The relationship between FTSWt, TTSW and OA, and their genetic control remains poorly understood. To our knowledge, there have been no reports yet of determination of the genetic regions responsible for FTSWt. Marguerit *et al*. (2012) reported the results of QTL analysis for acclimation of transpiration rate to water deficit in grapevines for different levels of FTSW: 60%, 40% and 20%. They also reported QTL mapping for TTSW, but no other results for QTL mapping for this trait were mentioned. QTL mapping for TTSW in sunflower has never been reported. In addition, although OA is receiving increasing recognition as a major mechanism of dehydration and drought tolerance (Flower and Ludlow, 1986; Saranga *et al.,* 2004), and genetic analysis for OA has been reported by numerous authors in a wide range of crops (Morgan, 1984; Zhang *et al*., 1999; Serraj and Sinclair, 2002), QTL mapping for OA associated with dehydration and drought tolerance in sunflower is rarely explored

(Poormohammad Kiani *et al.,* 2007b). Therefore, the objective of this paper was to investigate the patterns of genetic variation of control of transpiration (FTSWt), water extraction capacity (TTSW) and dehydration tolerance (OA), as well as their genetic control.

Materials and Methods

Plant source

One hundred and forty eight F8 recombinant inbred lines (RILs) and their parents, XRQ and PSC8 (150 genotypes), were used in the experiment. XRQ and PSC8 are parental lines of the "INEDI" RIL population developed by INRA (Vincourt *et al*., 2012) and both XRQ and PSC8 behaved differently in response to water deprivation (Rengel *et al*., 2012).

Experimental setup and progressive drought stress treatment

A randomized complete block design with three replicates was used for the progressive drought stress treatment (three replicates X 150 genotypes = 450 plants) called ws. There was another replicate (150 plants) that was considered as a well-watered treatment, called ww. In total, there were 600 plants.

The plants were sown in two-liter pots that contained a mixture of 50% soil (collected from the field), 30% organic matter and 20% sand. The pots were arranged in a greenhouse at the INRA Auzeville station, Toulouse, France (43°31'46,94" N; 1°29'59,71" E). Greenhouse air temperature was set at 25/18 $\pm$ 2^0C (day/night) and relative humidity was 55-75%. The pots were arranged on 100 balances (maximum capacity 30 kg, precision 2 g, model SXS, GRAM, Spain), with six pots per balance. Each pot was covered with a 3 mm layer of polystyrene sheet to prevent the evaporation of water from the soil surface.

At 1 day after emergence (DAE) (17 days after sowing), all 600 pots were watered to field capacity, by fully irrigating each pot and then allowing the water to drain for 24 h. At field capacity, the mean soil water content (SWCfc) in the pots was 39.5%. The 600 pots were then kept without irrigation for 17 days. Starting at 17 DAE, we maintained the ww treatment (150 plants) at 30% of SWC (well-watered conditions but not saturation) by daily irrigation. The ws treatment (450 plants) was kept without irrigation until harvest, when the permanent wilting point was reached and the SWC was measured (SWCwp). The permanent wilting point was reached on the same date for all genotypes (at 32 DAE $\pm$ 1 day).

Trait measurements

Total transpirable soil water (TTSW) and threshold of fraction of transpirable soil water (FTSWt)

Throughout the experiments, the amounts of water in the pots were determined by weighing the pots every day. This weighing recorded the amount of daily water loss, corresponding to the daily transpiration of the plants. For each pot, at the end of the experiment, total transpirable soil water (TTSW) was calculated as follows.

$TTSW = PW_{fc} - PW_{wp}$ (Eq. 1)

where PW_{fc} was the initial pot weight at field capacity and PW_{wp} was final pot weight at wilting point. From these data, the soil water status in the pots for each plant can be determined each day, by calculating the soil water content (SWC) as follows .

$SWC = (PW_d - PW_{wp}) / PW_{wp}$ (Eq. 2)

In this study, we normalized SWC by using fraction of transpirable soil water (FTSW), as proposed by Sinclair & Ludlow (1986). The daily value of FTSW was calculated as the ratio of the amount of transpirable soil water still remaining in the pot to TTSW:

$FTSW = (PW_d - PW_{wp}) / TTSW$ (Eq. 3)

Transpiration of ws and ww plants was used to determine normalized transpiration ratio (NTR). Firstly, transpiration rate was calculated per unit leaf area by dividing the daily transpiration rate by the leaf area (LA). Secondly, the transpiration rate was normalized by dividing each transpiration rate of a ws plant (for each replicate) by the transpiration rate of a ww plant. This second normalization gave NTR, which accounted for plant-to-plant variation in transpiration within each genotype. Due to the large number of plants in the experiment, we estimated LA of the plants by using a computer image analysis system, winFOLIA (Regent Instruments, Quebec, Canada). The leaf images were obtained with a digital camera (Canon EOS400d), pictures were taken from above by using camera tripod.

The measurement of plant response to water deficit (traits related to the control of transpiration) used a regression approach to model individual plant response. The parameters from these models were used as quantitative traits in the association analysis. Two traits were estimated by using break-linear models: (i) FTSWt, the threshold of transpirable soil water (FTSW) at which the plant transpiration rate (NTR) began to decline, Equation 4, and (ii) SWCt, the value of soil water content (SWC) when the plant transpiration (NTR) rate was zero, Equation 5 and 6.

(i)

If $x < a$, $NTR = \frac{0.9}{a} \times x + 0.1$

else, $NTR = 1$ (Eq. 4)

where x was FTSW, and a was FTSWt.

(ii)

If $x < a$, $NTR = \frac{1-b}{a} \times x + b$

else, NTR =1 (Eq. 5)

where x is SWC, a is SWCt, the x-intercept was computed as:

$$x0 = -b \times \frac{a}{1-b} \quad \text{(Eq. 6)}$$

Leaf osmotic potential (OP) and adjustment (OA) measurements

When ws plants began to wilt (two days before the permanent wilting point was reached), the uppermost expanded leaf was used for measurement of leaf osmotic potential (OP). The leaf samples for ww plants were taken at harvest.

The leaf sample that was used for OP measurement was half of the uppermost fully expanded leaf of each individual plant (without petiole). Before measuring OP, the leaf samples were rehydrated in distilled water for 24 h at 4^0C in a dark room. This was done to ensure that OP was measured at full turgor (called OP_ws for the water-stressed plants and OP_ww for the well-watered plants). After rehydration, the osmotic potentials of leaf samples were measured on expressed sap using 10 µl aliquots placed in an osmometer (Wescor, model 5520, Logan, UT, USA) calibrated with manufacturer's solutions. OP was then determined from the osmometer reading (in mmol kg^{-1}) using the Van't Hoff relation:

OP = –RTd X c /1000 (Eq. 7)

where R is the gas constant, T is temperature in ºKelvin, d is density of water at temperature T, and c is concentration of osmotically-active solutes, given by the osmometer. Osmotic adjustment (OA, MPa) was then determined using the following equation:

OA = OP_ww - OP_ws (Eq. 8)

where the value of OP_ws was represented by the mean of three replicates.

Genetic map construction

The genetic map consisted of 2610 markers located on the 17 LG for a total genetic distance of 1863.1 cM and grouped on 999 different loci. The gDNA from the INEDI RILs population obtained from the cross between XRQ and PSC8 lines (210 samples) were genotyped with the Infinium array. All genotyping experiments were performed by Integragen (IntegraGen SA, Genopole Campus 1 - Genavenir 8, 5 rue Henri Desbruères, 91000 Evry, France.) and the

genotypic data were obtained with the Genome Studio software (Illumina) with automatic and manual calling. A set of 9832 SNPs were used to produce an Infinium HD iSelect BeadChip (Infinium). These SNPs were selected from either genomic re-sequencing or transcriptomic experiments. From the 9832 SNPs, 2576 were polymorphic between XRQ and PSC8. We used CarthaGène v1.3 (De Givry *et al*., 2005) to build the genetic maps. We added the genotypic data of markers from a consensus map (Cadic *et al*., 2013) to assign the Infinium SNPs to the appropriate LG.

Statistical analysis and QTL mapping

The software of statistical package PASW statistics 18 (IBM, New York, USA) was used to analyze genotype and replicate effects by analysis of variance (ANOVA) and to estimate phenotypic correlation by Pearson's correlation. Means of the traits were compared using a Student-Newman-Keuls (SNK) test ($P < 0.05$). For the FTSW and SWC threshold (FTSWt and SWCt) analysis, R software (R Development Core Team, 2012) was used. Each NTR value was plotted against corresponding FTSW and SWC values. FTSWt and SWCt where NTR initiated its decline were determined using a plateau regression.

QTL mapping was carried out using MCQTL, software for QTL analysis (http://carlit.toulouse.inra.fr/MCQTL/). The MCQTL package is comprised of three software applications. The first component, TranslateData reads data from MAPMAKER (Lincoln *et al*., 1993) -like files. The second component, ProbaPop computes QTL genotype probabilities given marker information at each chromosome location for each family and stores them in XML formatted files. The last component, Multipop builds the pooled model using the genotype probabilities, computes Fisher tests and estimates the model parameters (Jourjon *et al*., 2005). Significant thresholds ($P < 0.05$) for QTL detection were calculated for each dataset using 1000 permutations (Churchill & Doerge, 1994) and a genome-wide error rate of 0.01 (Type I error). The corresponding type I error rate at the whole-genome level was calculated as a function of the overall number of markers in the map and the number of markers in each linkage group.

Results

Phenotypic analysis for FTSWt-related traits

We examined the response of NTR to soil drying, i.e. decreasing FTSW and SWC. The overall response for all genotypes in transpiration rate to soil drying fitted the general pattern represented by two linear slopes (Fig. S1 and S2). NTR at high FTSW or SWC was defined

by a plateau, and at FTSW or SWC below a threshold (FTSWt or SWCt), NTR decreased linearly with further decreases in FTSW or SWC.

The relationship between NTR vs. FTSW and SWC for XRQ and PSC8 (Fig. 1) shows that there was variability in the threshold for the decline in NTR between genotypes. These two genotypes represent two contrasting examples in the FTSWt and SWCt values between 0.35 and 0.44, and between 0.22 and 0.25 for FTSWt and SWCt, respectively.

Statistics explaining phenotypic variability of all traits and the mean square of genotype from the analysis of variance (ANOVA) test are given in Table 1. Results of the ANOVA test showed the large effects of genotypes ($P < 0.001$) for FTSWt, SWCt, TTSW and OP_ws. Phenotypic correlations for all traits are shown in Table 2.

Table 1 Phenotypic variability of all studied traits

Traits	Minimum	Maximum	Mean	Std.deviation	MSg
FTSWt	0.15	0.47	0.31	0.05	0.009***
SWCt	0.16	0.34	0.21	0.02	0.003***
TTSW	370	528	470	27	3742.332***
OP_ws	-1.75	-1.36	-1.51	0.07	0.052***
OP_ww	-0.94	-0.52	-0.74	0.09	
OA	0.51	1.02	0.77	0.11	

*** Significant at $P < 0.001$. FTSWt, threshold of the fraction of transpirable soil water; SWCt, threshold of the soil water content; TTSW, total transpirable soil water (ml); OP_ws, leaf osmotic potential at water-stressed condition (MPa); OP_ww, leaf osmotic potential at well-watered condition (MPa); OA, osmotic adjustment (MPa). MSg, mean square of genotypes that calculated by analysis of variance, anova.

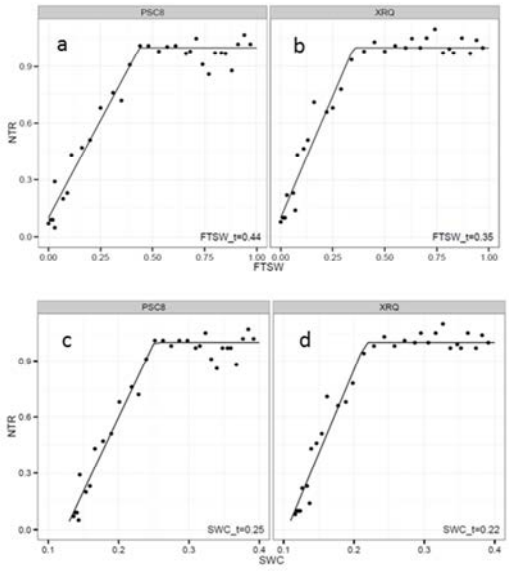

Fig. 1 NTR response to the FTSW (a,b) and SWC (c,d) of parental genotypes (PSC8 and XRQ). Dot symbols are mean of measured data and curves represent the results of linear regression. FTSWt and SWCt at which NTR began to decrease are shown.

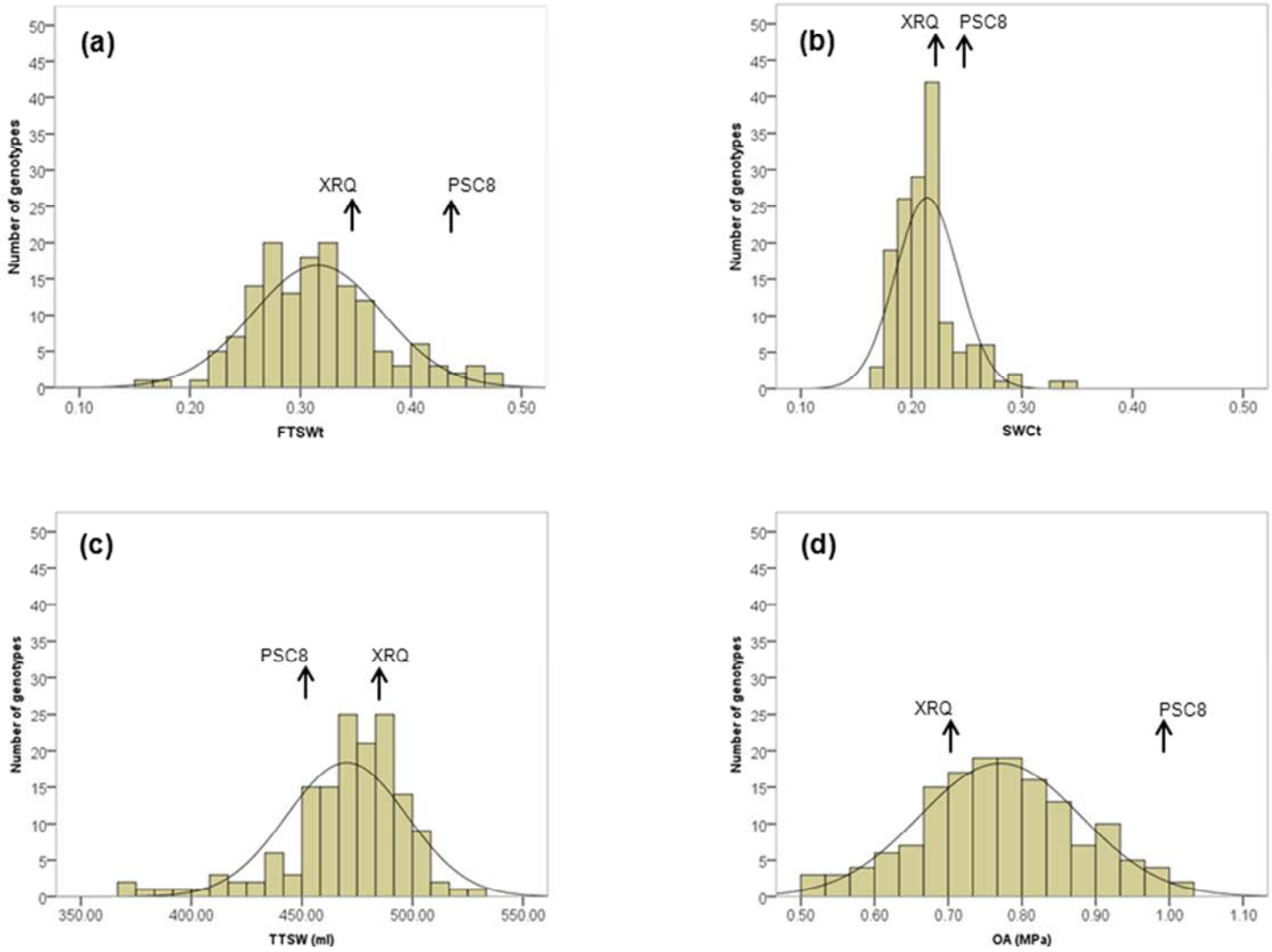

Fig. 2 Genotype distribution for FTSWt (a), SWCt (b), TTSW (b) and OA (d) of 150 genotypes (148 RILs and two parental lines). Parentals mean are indicated in each genotype distribution of the trait.

Table 2 Correlation between FTSWt, SWCt, TTSW, OP_ws, OP_ww and OA

Trait [x]	FTSWt	SWCt	TTSW	OP_ws	OP_ww	OA
FTSWt		0.67***	-0.24**	-0.01[ns]	0.01 [ns]	0.01 [ns]
SWCt			-0.71***	0.14 [ns]	0.13 [ns]	0.01 [ns]
TTSW				-0.26**	-0.22**	0.01 [ns]
OP_ws					0.17*	-0.59***
OP_ww						0.69***

[x] The abbreviations of the traits can be seen in Table 1; * Significant at $P < 0.05$, ** Significant at $P < 0.01$, *** significant at $P < 0.001$,[ns] not significant.

Table 3 Significant quantitative trait loci (QTL) detected for FTSWt, TTSW, OP_ws, OP_ww and OA

Trait[x]	Chromosome	QTL name	QTL position (cM)	Nearest marker	R^{2a} (%)	Additive effect[b]
FTSWt	LG14	*ftsw$_t$*	27.9 (0-107.7)	ORS1079	6	+0.006
TTSW	LG06	*ttsw1*	64 (0-69.5)	HA015446_341	7	+8.163
	LG14	*ttsw2*	106.5 (100.1-107.7)	HA015659_281	11	-10.200
OP_ws	LG11	*op_ws*	3.8 (0-90.9)	HA005673_395	7	+0.0146
OP_ww	LG09	*op_ww*	59.6 (37.8-84.1)	HA007863_575	8	+0.0138
OA	LG10	*oa1*	111.1 (0-112.5)	HA011636_757	6	-0.0159
	LG11	*oa2*	5.8 (0-98.4)	HA006174_145	5	-0.0156

[x]The abbreviations of the traits can be seen in Table 1; [a]Phenotypic variance explained by QTL effect; [b]Additive effect estimated as one-half the difference in homozygotes carrying either allele of parents (XRQ or PSC8), positive values indicate that XRQ allele increases the trait value, while negative values indicate that PSC8 allele increases the trait value.

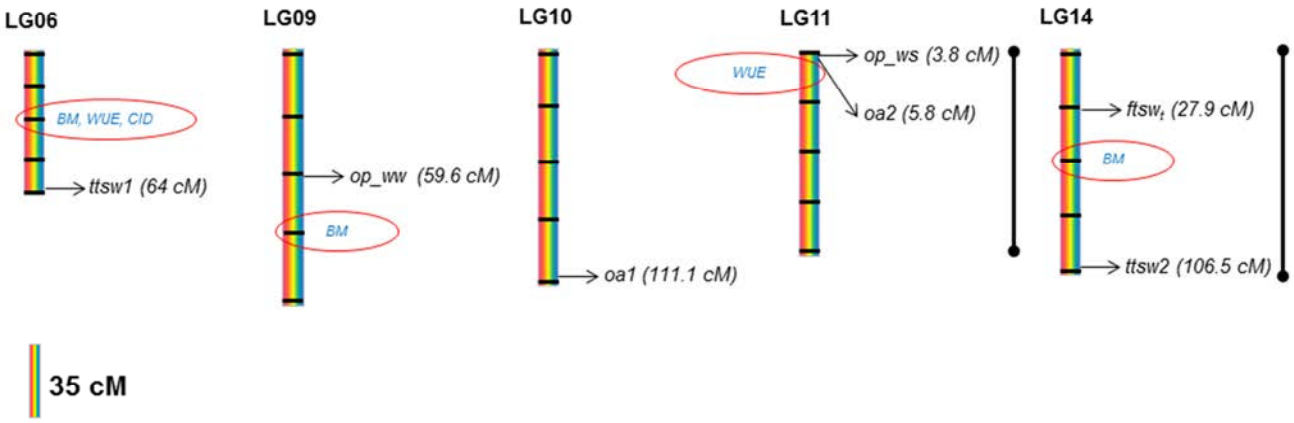

Fig. 3 Locations of QTL for FTSWt, TTSW, OP_ws, OP_ww and OA on the genetic map. Black lines on each linkage groups (LG) indicate the markers position (distance in centimorgan, cM) at the top, before-middle, middle, after-middle, and bottom of LG (from lower position at the top LG and upper position at the bottom LG). Vertical black lines (bars), besides of LG11 and LG14 indicate interval position (inferior and superior) of the two different QTL. QTL for BM (biomass), WUE (water use efficiency), and CID (carbon isotope discrimination) are also indicated (Adiredjo et al., 2014).

We provide distributions of RILs means for FTSWt, SWCt, TTSW and OA that are following a normal law (Fig. 2). RILs extremes for the traits were commonly exceeded by either parent, indicating transgressive segregation, particularly for FTSWt and TTSW. Therefore, in terms of phenotypic variability control of the transpiration trait, FTSWt varied more than SWCt.

Quantitative trait loci (QTL) identification

Seven QTL were identified in the present study, with one and two QTL having been identified for FTSWt and TTSW respectively, and four QTL having been identified for OP and OA (one QTL for each of OP_ws and OP_ww, and two QTL for OA) (Table 3). We detected a positive additive effect of QTL for FTSWt, and both positive and negative effects of QTL for TTSW (QTL on chromosome LG06 and LG14). The positive additive effects of QTL were also observed for OP_ws and OP_ww. In contrast, the two negative additive effects of QTL were shown for OA (QTL on chromosome LG10 and LG11). The percentage phenotypic variance explained by the QTL (R^2) ranged from 5% to 11%, with six of the seven QTL having variance of less than 10%.

The positions of the QTL are given in Fig. 3, and the likelihood odds ratio (LOD) profiles generated using MCQTL are given in Fig. 4. No QTL were detected for SWCt. The QTL for FTSWt co-localized with the QTL for TTSW (mapped on chromosome LG14, with interval position at 0-107.7 cM, Table 3) whereas the QTL for OP_ws co-localized with the QTL for

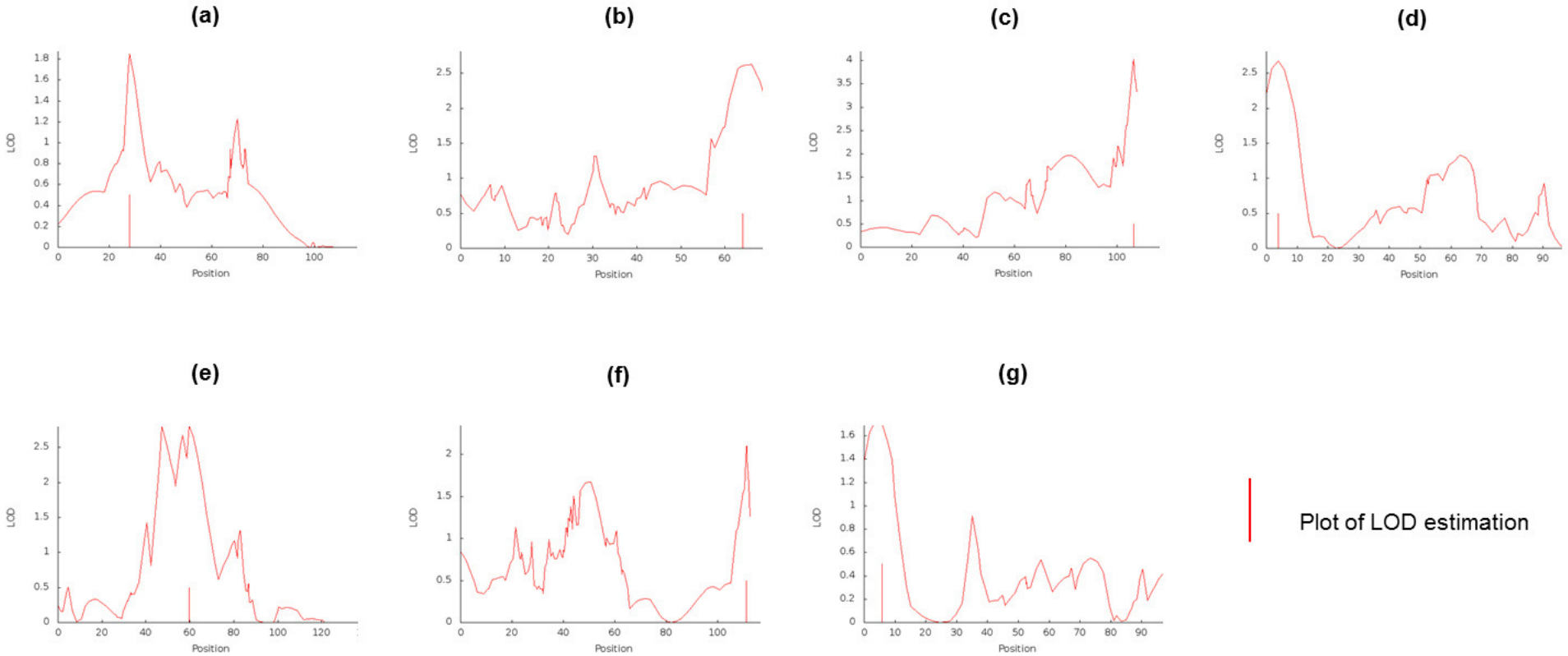

Fig. 4 Likelihood odds ratio (LOD) positions on the genetic map showing the locations of QTL controlling the studied traits identified by MCQTL. These figures present the QTL of *ftsw*$_t$ on LG14 (a), *ttsw1* on LG06 (b), *ttsw2* on LG14 (c), *op_ws* on LG11 (d), *op_ww* on LG09 (e), *oa1* on LG10 (f), and *oa2* on LG11 (g).

OA (mapped on chromosome LG11, with interval position at 0-98.4 cM). No significant interaction (co-localization) was detected between the QTL for FTSWt and OP, OA, as well as between the QTL for TTSW and OP, OA.

Discussion

FTSWt-related traits and phenotypic variability

Genotypic behavior of sunflower subjected to water deficit is particularly variable. It results from a combination of several forms of physiological behavior, such as the sensitivity of stomatal closure to SWC, the capability to extract water from the soil and the tolerance of the plant to dehydration. To our knowledge, using a large number of genotypes to explore genotypic behavior of sunflower under drought has not been reported in literature.

In our experiment, two-segment plateau regression was used to determine a breakpoint, the FTSW threshold (FTSWt), where NTR began to decline (see Fig. S1). FTSWt reflects the point at which stomata begin to close and then photosynthesis begins to decline (Miller, 2000). FTSWt has been reported for crop plants (Sinclair *et al*., 1995), and specifically for sunflower (Sadras & Milroy, 1996; Casadebaig *et al*., 2008) in a small number of genotypes. From our results, two main and extreme forms of genotypic behavior are highlighted: (i) a "conservative" strategy, where the plants react to drought stress by closing their stomata when FTSW is still relatively high, and (ii) a "productive" strategy, whereby the crop keeps transpiring despite increasing drought (Sinclair & Muchow, 2001). Between these two extreme forms, genotypes had a wide range of thresholds (from 0.15 to 0.47). PSC8, with FTSWt of 0.44 was a typical conservative genotype, while XRQ with FTSWt of 0.35 was intermediate between conservative and productive genotypes (Fig. 1a,b). The higher FTSWt value for PSC8 rather than XRQ in this study was in agreement with Rengel *et al*. (2012).

The phenotypic variability of transpiration control by sunflower RILs in this study was not in accordance with the anisohydric behavior which is usually attributed to this species (Tardieu & Simonneau, 1998). Anisohydric species typically display less sensitivity to stomatal closure in drying soil, which is represented by low FTSWt values, than isohydric ones (Jones, 2013). Nevertheless, we observed that the maximum FTSWt value in our RILs population was slightly smaller than the maximum FTSWt value of Casadebaig *et al*. (2008) using other sunflower genotypes (including commercial hybrids). They reported that the highest FTSWt value reached was 0.63 for a sunflower commercial hybrid, thus making it closer to isohydric behavior. Our results and those of Casadebaig *et al*. (2008) indicate that the investigated genetic variability in our experiment covers both anisohydric and isohydric behaviors. This was in accordance with an argument of Schultz (2003) and Jones (2007) that

different genotypes within a crop species, and even the same genotypes grown in different environments, can exhibit both response types.

In our study, soil water extraction capacity of the plant was determined by TTSW (Sinclair & Ludlow, 1986). We observed that phenotypic variability of TTSW was quite wide, from 370 to 528 ml. In this respect, our result was similar to Chimenti *et al*. (2002) but not Casadebaig *et al*. (2008). The potential soil water extraction has been previously studied in crop plants (Passioura, 1983; Monteith & Greenwood, 1986) and in sunflower on a range of soils (Meinke *et al*., 1993). However, we know little about the ability of roots to extract water from the soil. Three main ways can be explored: (i) higher root length density in the soil leading to greater soil moisture extraction involving osmotic adjustment in plants subjected to drought (Chimenti *et al*., 2002), (ii) root hydraulic resistance is often adjusted by the size of the root system (Steudle, 2001), (iii) large variations in hydraulic resistance in plants may be caused by cavitation in root xylem (Byrne *et al*., 1977) as well as shoot xylem (Salleo *et al*., 2000). These ways have to be explored to better understand how plants extract water from the soil and then use this physiological behavior to select genotypes that are more tolerant to drought.

Results of OP values were in the range of OP values of other crops. Commonly, the osmotic potential of most crop plants subjected to drought is between -1.5 and -2 MPa (Kramer, 1983; Serraj & Sinclair, 2002). We found wide phenotypic variability for OP_ws as well as OP_ww in our sunflower RILs (Table 2), suggesting that OP at full turgor can be used to characterize plant response to drought (Jones, 2013). Drought stress frequently affected and caused an increase in the quantity of osmotically active solutes, implying osmotic adjustment (OA). Phenotypic variability for OA in this study was also wide, and higher than OA values previously reported by Poormohammad Kiani *et al*. (2007a) using another sunflower RILs population. Substantial genetic variation has been reported for OA in major crop species with a range from 0.1 to 1.7 Mpa (Zhang *et al*., 1999). Therefore, the phenotypic variability of OP and OA in our experiment represented a substantial variability of tolerance to dehydration among the sunflower RILs.

Phenotypic correlations among traits

TTSW was negatively correlated either with FTSWt or with SWCt, indicating that genotypes which attempt to maximize water use by extracting more soil water were those having lower stomatal conductance sensitivity to drought ("productive" strategy). In contrast, no relationship was observed between OP and FTSWt-SWCt or between OA and FTSWt-SWCt. According to Comstock (2002), plants can close their stomata even without a reduction in

osmotic potential. In another words, the stomata may also be closed without leaf dehydration (Farooq *et al*., 2009). This due to hydraulic signaling through reduced hydraulic conductance. The reduction of hydraulic conductance which was caused by soil drying has reduced stomatal conductance. This is triggered by root-to-shoot signaling, such as an increased absicic acid (ABA) concentration (Jones & Tardieu, 1998). Another argument has been reported by Jones (2013), namely that the relationship between OP and stomatal conductance is largely coincidental, because OP tends to decline as the soil dries, while the key signal controlling stomatal closure in response to drought comes from the roots,

A significant negative correlation was observed between TTSW and OP (both OP_ws and OP_ww). This indicates that genotypes which were able to extract water efficiently from the soil are those with a lower osmotic potential in the leaves and thus a higher tolerance to dehydration. The decrease in OP in response to water deficit is a well-known mechanism whereby many plants adjust to drought conditions (Morgan, 1984; Patakas & Noitsakis, 1999). In contrast, no correlation was found between TTSW and OA, meaning that the capacity to adjust was independent of the osmotic potential at full turgor or the SWC. Therefore, sunflower RILs acclimation to drought was not due to OA. However, further investigation is required because inconsistent results in the literature have been reported: Chimenti *et al*. (2002) reported a positive association between soil water extraction (TTSW) and OA in a set of 25 sunflower RILs while Champoux *et al*. (2005) reported a negative association between soil water extraction capacity and OA for a drought-susceptible rice cultivar (Co39). Furthermore the OP_ws was negatively correlated with OA, suggesting that the plants which respond to declining osmotic potential under drought tend to be more tolerant to dehydration. OA refers to the lowering of OP due to net accumulation of solutes in response to water deficit, and results in the maintenance of a higher turgor potential.

Genetic control of FTSWt-related traits

In the present study, transgressive segregation was detected in the mapping population for all traits. It was mainly observed for FTSWt whose minimum and maximum values were extremely and significantly different from those of the parental lines (see Fig. 1a). Genetically, transgressive segregation can result from the expression of rare recessive alleles (Rick & Smith, 1953) or from complementary gene action (Vega & Frey, 1980). Since we used RILs population in our work, the transgressive segregation had to be due to complementary gene action (Grant, 1975; Vega & Frey, 1980).

By using numerous genotypes (150 genotypes) with progressive water stress, we could determine the genetic architecture of plant responses to soil water deficit through FTSWt,

TTSW, OP and OA. A relatively small number of QTL was detected, some of which were close to the detection limit (only one QTL for TTSW on chromosome LG14 accounting for > 10% of the phenotypic variance), suggesting that there are more QTL with small effects controlling these traits (Burke *et al.,* 2002; Brouillette *et al*., 2007).

A QTL for FTSWt co-localized with QTL for TTSW. In contrast, no significant QTL was identified for SWCt, although a strong positive phenotypic correlation was found between SWCt and TTSW. Although no QTL was found for SWCt, this result suggests that the control of stomatal closure (plant response to soil water deficit) was closely linked to the soil water extraction capacity of plants.

We observed that the genetic control of FTSWt-TTSW and OP-OA was completely independent, as no co-localization of QTL was observed for these soil water and plant water status-related traits. This was consistent with our phenotypic data showing that no significant correlation was found between the traits, except between TTSW and OP.

We compared the QTL position identified in the present study with the results obtained by Poormohammad Kiani *et al*. (2007b, 2009) for leaf water status-related traits using a different mapping population under well-watered and water-stressed conditions. According to the authors, one of the QTL controlling OP under well-watered conditions is located on chromosome LG09, and one of the QTL controlling OP under water stress is located on chromosome LG11. Their findings were similar to our results where the QTL for OP_ww and OP_ws were identified on chromosome LG09 and LG11, respectively. This suggests that multiple populations are needed for a wide range QTL detections and genotypic distinction among breeding materials (Hao *et al.,* 2009; Xing *et al*., 2012).

Lastly, we also observed a partly common genetic basis for plant responses to soil water deficit, productivity (biomass) and water use efficiency by comparing the QTL mapping results in this paper with our previous study (Adiredjo *et al*., 2014). From our previous study, QTL for biomass and water use efficiency were identified on chromosome LG06 as well as on chromosome LG14 (QTL for biomass). In the present study, the QTL for FTSWt and TTSW were identified on those chromosomes. These findings suggest that the genetic control of FTSWt and TTSW is dependent on biomass and water use efficiency. Therefore, detailed characterization of these genomic regions may lead to an improved understanding of drought resistance and might set the stage for the positional cloning of drought resistance genes (Poormohammad Kiani *et al*., 2009).

Conclusions

This is the first QTL mapping of transpiration control by using FTSWt in crop plants to show a link between genetic variation in the FTSWt and TTSW. Genotypes that closed their stomata under severe drought or low SWC were also those with a high TTSW. The inter-relationships between FTSWt and TTSW, as well as between OP and OA, were consistently shown by the QTL co-localization. None of the QTL for FTSWt-TTSW and OP-OA co-localized, indicating an independent genetic control between dehydration tolerance (OA) and either control of transpiration (FTSWt) or water extraction capacity (TTSW).

It is also the first report on QTL mapping for TTSW in sunflower. The genetic control of TTSW was closely linked to biomass, water use efficiency and carbon isotope discrimination, rather than to OA. This work also suggests new avenues through which to investigate the genetic controls over FTSWt, TTSW and OA, to thus narrow the range of candidate genes underlying a QTL.

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Author contributions

Designed and performed the experiments: ALA, PG, TL. Analyzed the data: ALA, PG, NL. Analysed and wrote the NTR modelisation: PC, ALA, PG. Contributed materials/analysis tools: NL, ALA, PG. Wrote the paper: ALA, PG.

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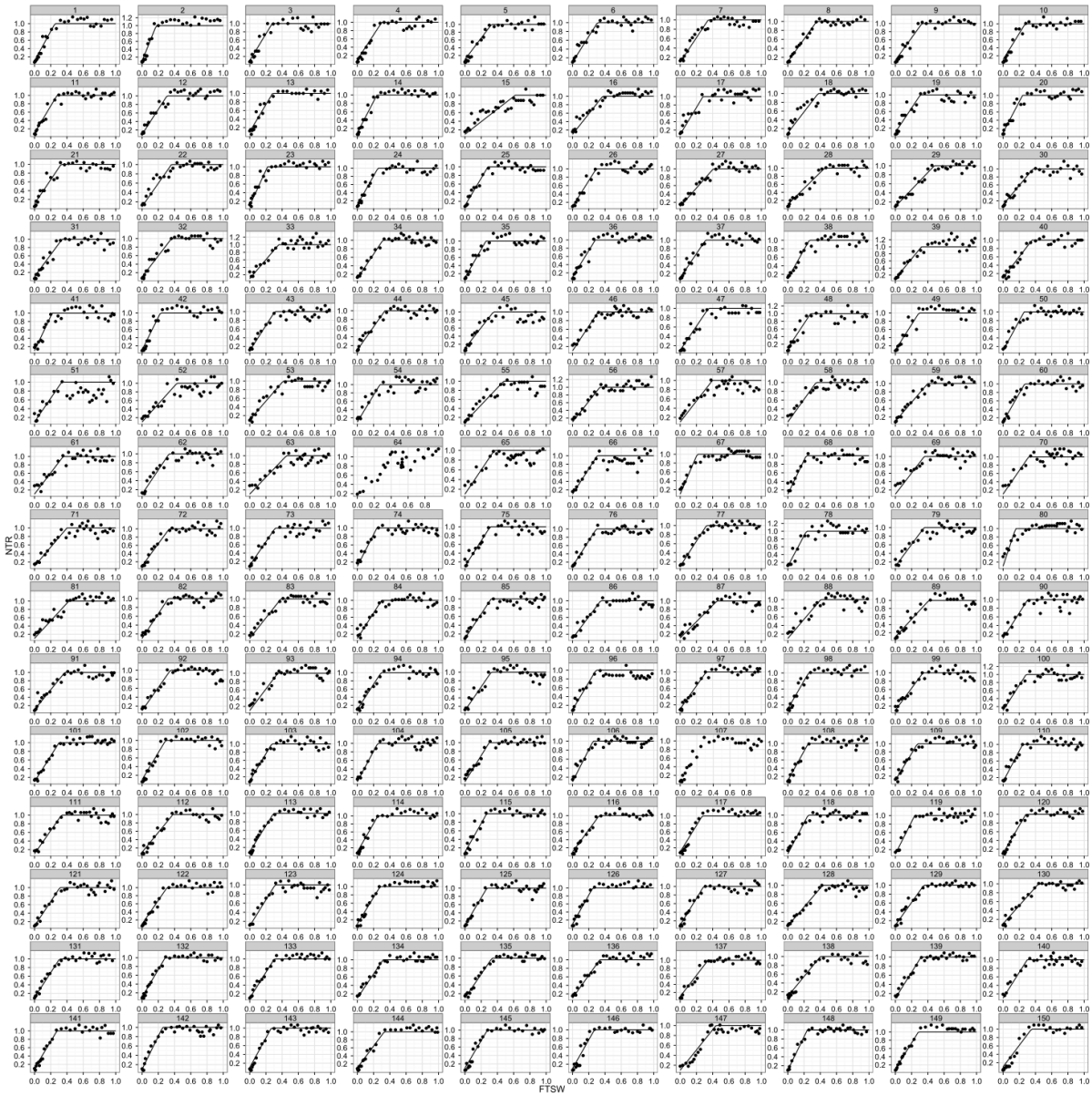

Fig. S1 Normalized transpiration ratio (NTR) response to the fraction of transpirable soil water (FTSW) among 150 genotypes (148 RILs and two parental lines). Data represent the mean of three replicates.

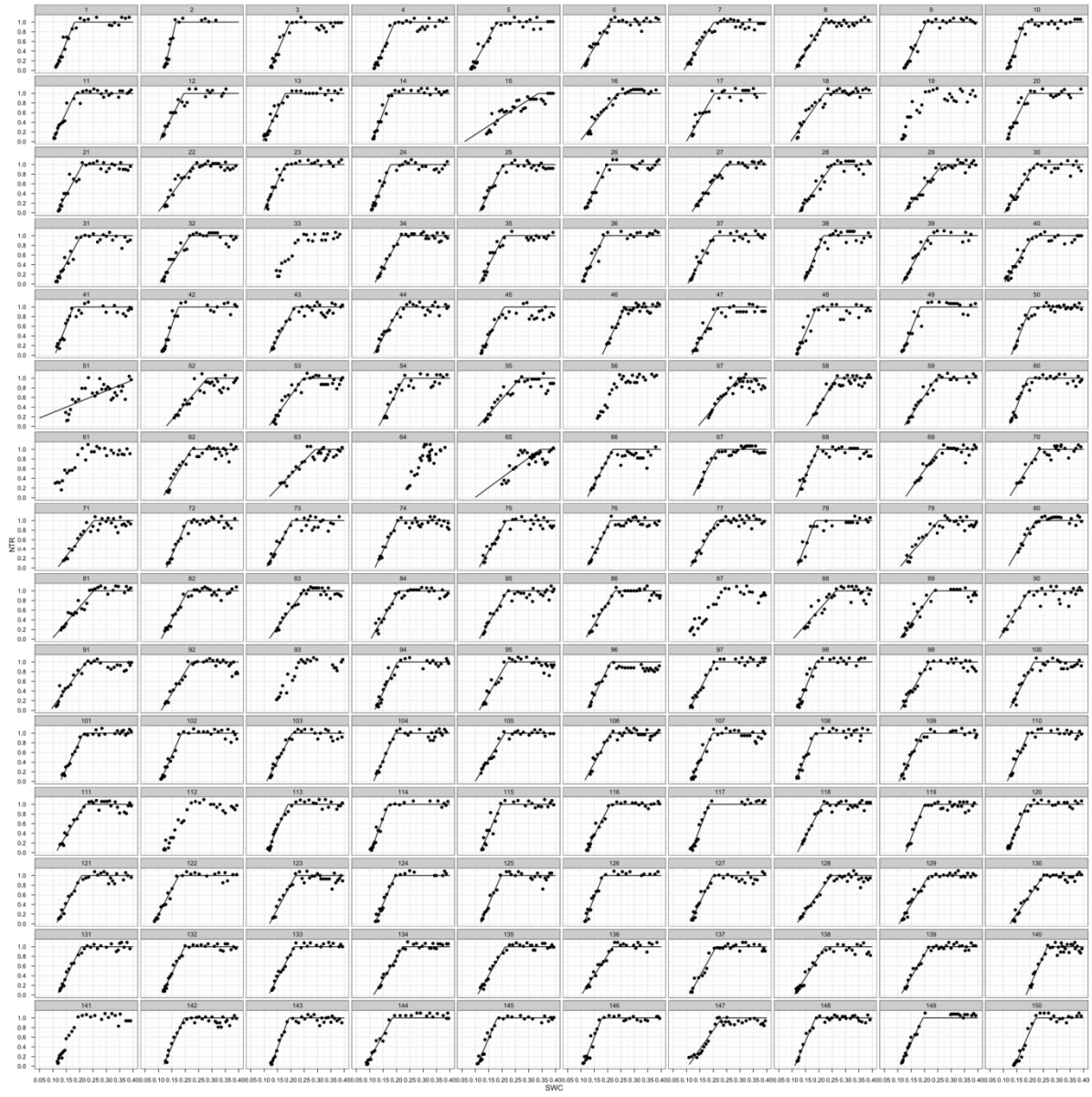

Fig. S2 Normalized transpiration ratio (NTR) response to soil water content (SWC) among 150 genotypes (148 RILs and two parental lines). Data represent the mean of three replicates.

The results of the first and second articles (Section 4.1 and 4.2) highlight the genetic control of WUE and CID, as well as the genetic control of plant-water relation traits, i.e. transpiration control, water extraction capacity, and dehydration tolerance. Besides, overall result indicated that CID can be used to improve WUE by using marker-assisted selection (MAS) approaches and this trait was genetically associated with the plant-water relation traits. In the context of plant water-relation traits, variability of water extraction capacity (expressed as TTSW) and root hydraulic conductance, especially in sunflower, is rarely reported in the literature. Therefore, it is required to do an experiment to evaluate root hydraulic properties in several sunflower genotypes differing in WUE (or CID). The experiment was conducted as a preliminary approach (only in well-watered condition).

4.3 Hydraulic conductivity and contribution of aquaporins to water uptake in roots of four sunflower genotypes

Afifuddin Latif Adiredjo[a,b], Olivier Navaud[c], Philippe Grieu[a,x], Thierry Lamaze[c,x,*]

[a]Université de Toulouse, INP - ENSAT, UMR 1248 AGIR (INPT-INRA), BP 52627, 31326 Castanet-Tolosan, France
[b]Brawijaya University, Faculty of Agriculture, Department of Agronomy, Plant Breeding Laboratory, Veteran street, 65145, Malang, Indonesia
[c]Université de Toulouse, UPS - Toulouse III, UMR 5126 CESBIO, 18 avenue Edouard Belin, 31401 Toulouse Cedex 9
[x]Ph.D. supervisors of the first author

Submitted to Botanical Studies

* Corresponding author at: Université de Toulouse, UPS - Toulouse III, UMR 5126 CESBIO, 18 avenue Edouard Belin, 31401 Toulouse Cedex 9, France.
Tel.: +33 5 61 55 85 14; fax: +33 5 61 55 85 00
E-mail address: thierry.lamaze@cesbio.cnes.fr (T.Lamaze)

Abstract

This article evaluates the potential of intraspecific variation for whole root hydraulic properties in sunflower. We investigated genotypic differences related to root water transport in four genotypes selected because of their differing water use efficiency. We used a pressure-flux approach to characterize hydraulic conductance (L_0) which reflects the overall water uptake capacity of the roots and hydraulic conductivity (Lp_r) which represents the root intrinsic water permeability on an area basis. The contribution of aquaporins (AQPs) to water uptake was explored by using mercuric chloride ($HgCl_2$), a general AQPs blocker. Three conclusions emerge from our results: (i) a large variation in morphological and hydraulic profiles in sunflower, (ii) a varying contribution of AQPs to hydraulic conductivity between genotypes as indicated by the mean percentage of Lp_r inhibition after $HgCl_2$ treatment and (iii) root anatomy, which appears as a major determinant of the water transport properties of the whole organ, is able to compensate for a low AQP contribution. Overall our analysis points to marked differences between genotypes in the intrinsic aquaporin-dependent path but not in the intrinsic AQP-independent paths. Information on the hydraulic properties of root tissues and organs might have to be taken into account for plant breeding.

Keywords: sunflower, aquaporins, root, hydraulic conductivity.

Introduction

Terrestrial plants are dependent on essential leaf physiological processes such as a continuous supply with water since photosynthesis cannot be dissociated from transpiration. Water balance at the whole plant level should be regulated by coupled responses between the above-ground and below-ground parts (Shimizu et al. 2005). In the soil-plant-atmosphere continuum, the root offers the second largest resistance to water transport after the stomata (Steudle et al. 1987). Thus, information on the hydraulic properties of roots might be a key step for understanding whole-plant water relations and selection of water stress-resistant species or genotypes and therefore plant breeding (Sutka et al. 2011).

In roots in which the xylem vessels are fully developed, the resistance to water transport is located in the radial direction (Steudle and Peterson 1998; Ruggiero et al. 2003). Radial transport in roots occurs simultaneously through the cell to cell pathway and the apoplastic pathway. The cell to cell route is composed of the symplastic (through plasmodesmata) and the transcellular (involving crossing of membranes) paths. The apoplastic pathway is usually considered to have the least hydraulic resistance and is often be considered to be the main route (Heinen et al. 2009). However, the presence of lignified or suberized cell walls (casparian strips in root endodermis) which constitute apoplastic barriers forces water to cross cell membranes (Shimizu et al. 2005). Several studies have attributed an important role to the cell to cell path. Water movement through cell membranes is facilitated by water channels, called "aquaporins" (AQPs) (Maurel 2007). AQPs are integral membrane proteins that increase the permeability of membranes to water as well as other small molecules such as CO_2, glycerol and boron (Shimizu et al. 2005; Chaumont et al. 2005). AQP proteins contain thiol groups that are sensitive to $HgCl_2$ (Savage and Stroud 2007). Assuming that mercurial inhibition of water transport is via the inhibition of AQPs, the strength of inhibition may indicate the extent to which the cell to cell (transcellular) water movement (involving water passing through membranes) is involved in the radial transport of water across the root. Therefore, in order to divide radial water transport in roots into cell to cell and apoplastic pathways, $HgCl_2$ has been often used as a specific AQP inhibitor in crops, herbs and trees (Maggio and Joly 1995; Carvajal et al. 1996; Tazawa et al. 1997; Zhang and Tyerman 1999; Wan and Zwiazek 1999; North et al. 2004; Kamaluddin and Zwiazek 1999; Shimizu et al. 2005; Sutka et al. 2011).

Intraspecific root water transport has so far been compared in a small number of species: rice, maize, grapevine and *Arabidopsis* (Sutka et al. 2011). Sunflower is an economically important crop consumed worldwide. Although it is considered to be relatively tolerant to

water stress, sunflower production can be greatly affected by drought (Pasda and Diepenbrock 1990; Grieu et al. 2008). Indeed, although sunflower displays a high capability to extract water and then conduct it within the plant, the rate of leaf transpiration can reach very high values: up to 22 mmol H_2O $m^{-2}s^{-1}$ (Rawson et al. 1980). It has been shown that AQPs play a role in the sunflower response to drought at leaf and root levels (Ouvrad et al. 1996; Sarda et al. 1997, 1999). Information on the hydraulic properties of roots might be important for plant breeding. Thus, the aim of the present work was to evaluate variation in the root hydraulic properties of four sunflower genotypes selected because of their differing whole-plant water relations under well-watered conditions (Adiredjo et al. 2014). We used pressure-induced flow through root systems since the method has been widely employed to measure the hydraulic properties of roots from various plant species. The contribution of the AQP-dependent path (cell to cell path) to water transport was characterized using mercuric chloride (mercury) as an inhibitor.

Materials and Methods

Plant Source

Four recombinant inbred lines (RILs) of sunflower (*Helianthus annuus* L.) from the collection of the Laboratory of Plant-Microbe Interactions (LIPM), INRA of Toulouse, France, were used in the experiments, namely: RIL 043, RIL 127, RIL 149 and RIL 200. The four RILs are lines from the INEDI RIL population. This population was obtained by self pollination to at least F8 from a cross between XRQ and PSC8 (Vincourt et al. 2012). These parental lines have different drought tolerance behavior (Rengel et al. 2012). The four RILs were chosen on the basis of their differing water use efficiency (WUE) response under well-watered conditions, expressed as the ratio of aerial plant dry biomass to cumulative water transpired during vegetative growth, determined in a previous experiment: 1.86 $g.kg^{-1}$, 1.08 $g.kg^{-1}$, 2.65 $g.kg^{-1}$, 1.75 $g.kg^{-1}$ for RIL 043, RIL 127, RIL 149 and RIL 200, respectively (Adiredjo et al. 2014).

Plant culture, experimental design and root analysis

The plants were grown in a growth chamber (25°C/20°C in day/night) under 14 h of light (200 µmol m^{-2} s^{-1} photosynthetically active radiation at leaf level, Fluora, L 58 W/77, Germany) and 50 $\pm$ 5% RH in 250 mL pots. They were arranged in a randomized complete design with four RILs. To minimize the effects of heterogeneity within the growth chamber, the pots were rotated every week. In order to measure root hydraulics (conductance,

conductivity and contribution of AQPs), pressure-induced sap rates were determined on six-week-old sunflower seedlings, when above-ground parts were 15-20 cm high. The experiment was repeated three times.

Seedlings were grown in 250 mL glass pots of sand which could be easily washed and saturated with solution and then introduced into the pressure chamber. Thus the root system did not have to be excavated before the pressure-induced flow experiment and thereafter, excavation of the roots from the cultivated sand substrate could be gently achieved under water for determination of root characteristics. Upon completion of the exudation experiments, root fresh weight was determined. Then, properties i.e. root length, root surface area and volume of fine roots of each root system were determined with an image analyzer WinRHIZO 2007d (Regent Instruments, Quebec, Canada). Fine roots are the smallest diameter class (0 – 0.5 mm). Finally, root dry weight was measured.

Measurement of Root Sap Flow and $HgCl_2$ Treatment

For pressure-flow experiments, upon harvest, pots were washed three times (3 x 50 mL) to saturation with water for the control treatment and $HgCl_2$ solution (500 µM) for the inhibited treatment. Saturation of the pots allowed us to determinate root conductivity since under non-limiting soil moisture, plant resistance exceeds soil resistance (in wet soil, the bulk soil potential is close to 0 MPa (Ruggiero et al. 1999; 2003). Following this washing, the above-ground part was cut off with a razor blade just below the cotyledonary leaves (40-50 mm from the base). Pots with whole root systems were placed in a stainless steel pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA, Maggio and Joly 1995). Excised stems were sealed into the lid of the chamber through a silicone gasket so that part of the stem protruded and chamber pressure was gradually increased. Water expressed from each cut stem was collected using an Eppendorf tube containing dry cotton wool. The amount of sap was determined by weighing the tube before and after collecting the water. The sap flow (Jv), expressed as the quantity of water exuded from the cut stems, was monitored every 5 min for at least 45 min after it had reached a constant rate (less than 25 min). Aliquots of expressed sap were collected from each root system for later analysis of K^+ content.

In some experiments (pressure-flux curves), five pressures were applied in sequentially increasing order (0.1, 0.2, 0.3, 0.4 and 0.5 MPa) to whole root systems. Flow values were logged for 25 min at each pressure, allowing a 5-min equilibration period between pressures. Because the regression of sap flow on applied pressure was linear for all genotypes (data not shown), thereafter sap flows in control or $HgCl_2$-treated plants were determined at a constant

0.3 MPa pressure (Shimizu et al. 2005). Pressure gradually increased up to 0.3 MPa in the chamber, and was then held constant during the measurements (flow reached a steady-state value in about 20 min). The sap flow (Jv) is then used to define (i) the whole root hydraulic conductance (L_0) calculated as the sap flow rate per unit of pressure (µL s^{-1} MPa^{-1}) and (ii) the root hydraulic conductivity (Lp_r) calculated as the sap flow rate per unit of root surface and per unit of pressure (m s^{-1} MPa^{-1}).

We made experiments to determine the best method to treat the plants with the $HgCl_2$ inhibitor. For this we compared two methods. First, after measurement of pressure that induced sap flow in untreated roots, the pressure was released slowly in the chamber, then this chamber was opened, the cut stem was removed from the gasket and the pot was washed with $HgCl_2$ solution. The stem was sealed again in the gasket secured to the lid and the chamber was pressurized again to 0.3 MPa. However, this method was a delicate procedure that often caused damage to the stem. Second, measurement of the pressure that induced sap flow was done on distinct root systems, untreated and treated (control and $HgCl_2$). Therefore, calculation of the depressive effect of $HgCl_2$ on Jv was finally achieved by considering the second method rather than the first, since it was checked that the two methods gave similar results (data not shown). Experiments using the second method were performed three times involving six plants per RIL each time.

After washing the pot with $HgCl_2$, maximal inhibition was achieved in less than 40 min. The reversibility by 10 mM mercaptoethanol (ME) of inhibition by $HgCl_2$ of pressure-induced sap flow was evaluated following washing of the pot with 3x50 mL ME solution. The decrease by $HgCl_2$ was reversed by *ca* 90% by subsequent treatment of 30 min with ME (data not shown). Some sap samples collected from control or $HgCl_2$-treated de-topped plants were diluted (*ca* 40 µL of sap + 1 mL H_2O) and injected into a Dionex-D-100 ion chromatograph (USA). K^+ flux into the xylem was calculated as the product of the sap flux and concentration of K^+ in the sap. The flux of K^+ into the xylem was not significantly affected by the presence of $HgCl_2$ (5.08±0.10, 2.11±0.55, 1.76±0.11 and 6.72±1.23 µmol h^{-1} g^{-1} root fresh weight for RIL 043, RIL 127, RIL 149 and RIL 200, respectively, corresponding to 92.6%, 121.6%, 103.3% and 116.2% of the controls).

Statistics

Data were analyzed with the PASW statistics 18 (IBM, New York, USA) package. We used Least Significant Difference (LSD) test ($P < 0.05$) to make post-hoc comparisons between all means. Percentages of inhibition by $HgCl_2$ were calculated for each individual

root system, and mean values and standard deviations were calculated for the three experiments. In total, the response of nine $HgCl_2$-treated plants was compared with those of untreated plants for each genotype.

Results

Size of Root Systems

Morphological parameters of sunflower root systems are presented in Table 1. RIL 043 and RIL 127 had the largest and the smallest root systems for every parameter (fresh or dry mass, surface, length and fine root volume), respectively. Differences could reach 100% of the values, demonstrating considerable variation in root morphology between genotypes. RIL 149 and RIL 200 showed intermediate root characteristics.

Variation of Root Hydraulic Properties

Figure 1 shows representative time courses of cumulative water movement obtained before (control) and after treatment of the root with $HgCl_2$ for the four sunflower RILs. Sap flow (Jv) from untreated or treated root system remained virtually constant throughout the 40 min measurement period. Pressure-induced sap flow was almost twice as high in RIL 149 than in RIL 127 whereas RIL 043 and RIL 200 gave intermediate flows. The sap was strongly inhibited by $HgCl_2$ treatment in all genotypes (more than 50%).

Figure 2 presents mean values of L_0 and Lp_r for the four RILs. Mean values showed significant variation between RILs. The values of L_0 and Lp_r have been found to range from 0.7 to 1.2 µL s^{-1} MPa^{-1} and 5.10^{-8} to 8.10^{-8} m s^{-1} MPa^{-1}, respectively. The ranking of RILs for L_0 was RIL 149>RIL 043>=RIL 200>RIL 127 but the ranking was changed for Lp_r: RIL 149> RIL 200>RIL 127> RIL 043. Differences between extreme values were above 60% in both cases.

Contribution of AQPs to Water Uptake

In our experiment, the contribution of AQPs to sap flow was explored using mercuric chloride. L_0 and Lp_r fell to 30-40 % of the control value and differences appeared between RILs (Fig. 2A and 2B). $HgCl_2$-treated root systems displayed markedly different L_0 values between genotypes (Fig. 2A) which ranked as follows: RIL 043>RIL 149>=RIL 200>RIL 127 (L_0 for RIL 043 was about 70% higher than for RIL 127). By contrast, Lp_r values were similar for all four RILs following $HgCl_2$ treatment (Fig. 2B).

The contribution of AQPs to Lp_r (AQPs involvement) expressed as the relative decrease in Lp_r induced by $HgCl_2$ treatment was the highest in RIL 149 (73%) and the lowest in RIL 043 (55%) while other RILs displayed an intermediate contribution (Fig. 3).

Table 1

Root morphological parameters of four sunflower genotypes grown in a growth chamber under controlled and well-watered conditions

RIL	*N*	RFW (g)	RDW (g)	RL (cm)	RS (cm^2)	RV^x(cm^3)
RIL043	23	7.92 ± 2.08	0.29 ± 0.06	1669 ± 331	210 ± 35	2.39 ± 0.74
RIL127	19	3.16 ± 0.68	0.17 ± 0.05	1191 ± 211	134 ± 31	1.05 ± 0.43
RIL149	20	5.23 ± 0.95	0.24 ± 0.07	1271 ± 264	161 ± 30	1.63 ± 0.42
RIL200	23	7.21 ± 1.86	0.27 ± 0.08	1247 ± 457	152 ± 40	2.01 ± 0.69

N: number of roots, RFW: root fresh weight, RDW: root dry weight, RL: root length, RS: root surface, RV: fine root volume.
The data represent all the roots of the non-inhibited plants (control) and inhibited plants ($HgCl_2$). Values are means and standard deviations.
[x] Fine roots are the smallest diameter class (0 – 0.5 mm) determined by the WinRHIZO analysis.

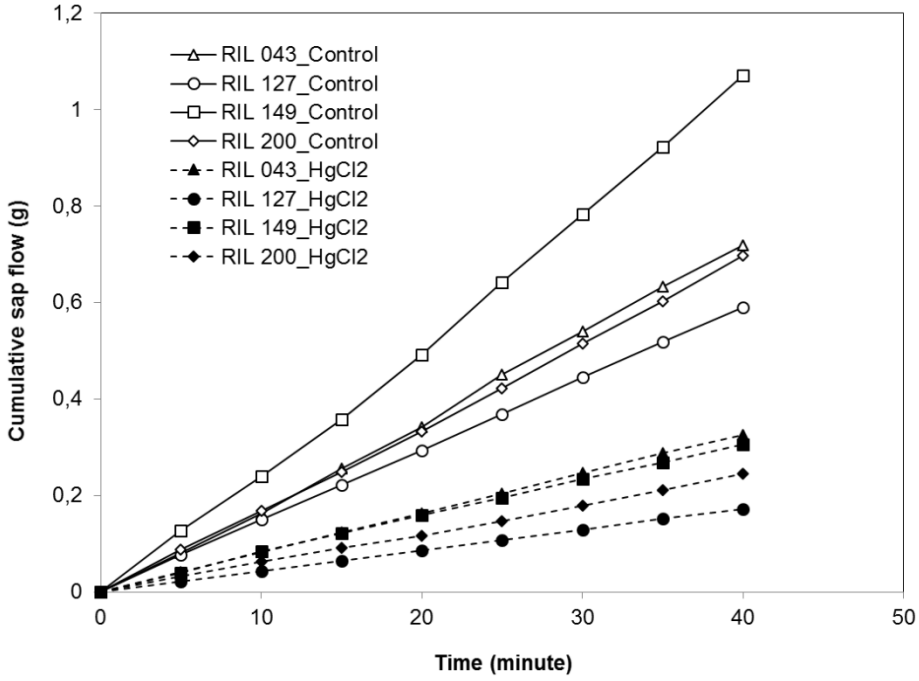

Fig. 1 Examples of cumulative sap flow for non-inhibited plants (control, open symbols) and inhibited plants ($HgCl_2$, solid symbols) of RIL 043, RIL 127, RIL 149, RIL 200. The cumulative data were obtained from eight successive, five minutes apart.

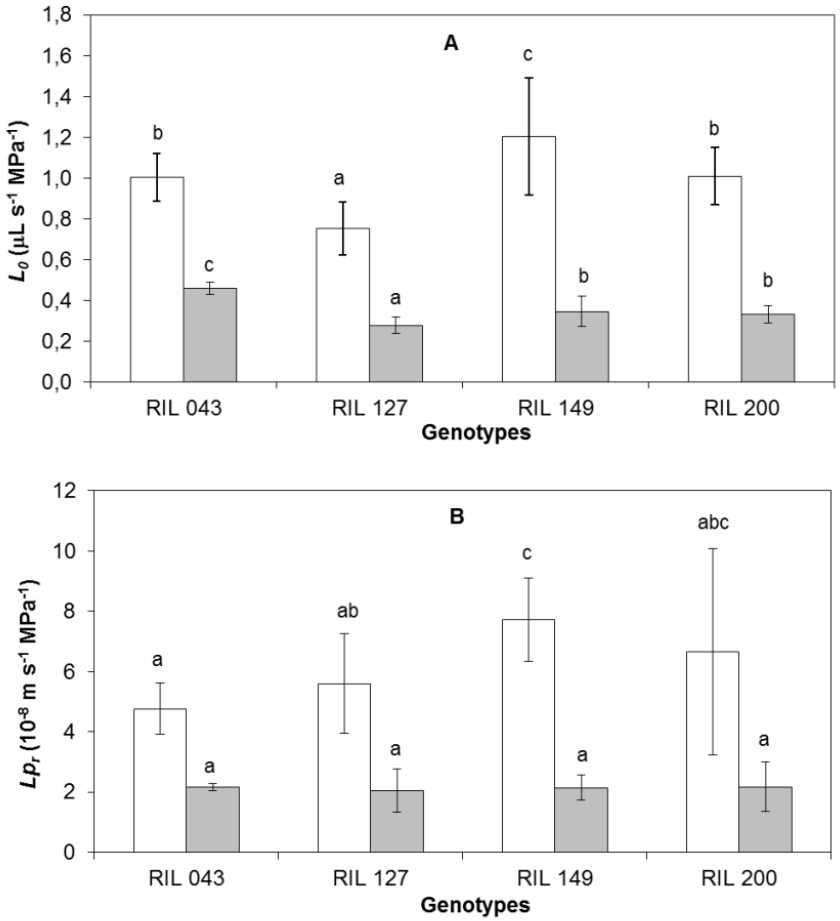

Fig. 2 Means of L_0 (A) and Lp_r (B) of RIL 043, RIL 127, RIL 149, RIL 200. The white bars are non-inhibited plants (control) and the black bars are inhibited plants ($HgCl_2$). Each value is the mean of nine plants ± standard deviation. Means within a treatment without a common letter are significantly different by $LSD_{0.05}$ test.

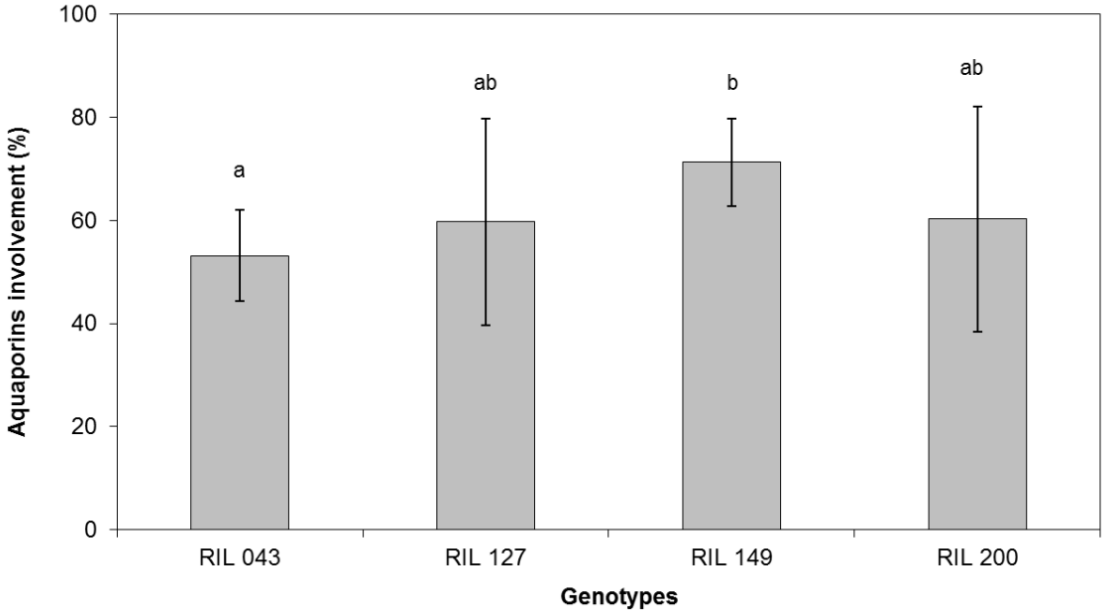

Fig. 3 Means of involvement of aquaporins of RIL 043, RIL 127, RIL 149, RIL 200. Each value is the mean of nine plants ± standard deviation. Means without a common letter are significantly different by $LSD_{0.05}$ test.

Discussion

In this study, the values of Lp_r for sunflower root systems ranged from 5.10^{-8} to 8.10^{-8} m s^{-1} MPa^{-1} and were within the range of values reported for other species (Liu et al. 2009; Sutka et al. 2011, Sakurai-Ishikawa et al 2011), although rather on the lower end of *Lpr* scale reported for roots of annual (crop) plants. Our values of sunflower root hydraulic conductivity were very similar to values reported for roots of 20-d-old sunflower plants (*ca* 600 µL h^{-1} g^{-1} root fresh weight MPa^{-1}, see Fig. 1 and Table 1, Quintero et al. 1999) but Alfalfa had Lp_r values 10 times higher than sunflower (Li et al. 2007). The pressure that induces flow through root systems has been considered by some authors (Li et al. 2007; Liu et al. 2009) to be inappropriate for characterizing "absolute" hydraulic values, because the externally applied pressure can induce flow through pathways external to the root system. Indeed, pressure chamber experiments give higher values of Lp_r than other methods using root pressure probes: resistance may vary according to the nature of the driving force for water movement (osmotic versus hydraulic) and the flow rate (Liu et al. 2009). It has been shown by Vandeleur et al. (2014) that shoot manipulation affected root hydraulic characteristics in grapevine, soybean and maize. Here, we used detopped plant to measure root hydraulics, thus a note of caution is warranted regarding the absolute values reported. However, the purpose of the present work was to compare the hydraulics of several sunflower RILs differing in their whole-plant water relations. Root *Lp* has been shown repeatedly to change with the volume flow rate through the root system (Sakurai-Ishikawa et al. 2011, Laur and Hacke 2013). However, quantitative comparison of the flow rates induced here through pressure gradient across roots (Fig. 1) with transpirational water loss rates of plants of the same age (850±22 µL h^{-1}, 785±19 µL h^{-1}, 1098±20 µL h^{-1} and 805±18 µL h^{-1} for RIL 043, RIL 127, RIL 149 and RIL 200, respectively, Adiredjo et al. 2014) clearly indicates that flow rates were similar in intact plants and in isolated root systems.

A $HgCl_2$-induced reversible inhibition of root water flow is consistent with the presence of a protein-mediated path for trans-membrane sap flow in the sunflower root. To avoid non-specific effects, concentrations have to be as low as possible and time of exposure as short as possible. Coskun et al. (2012) recommended caution in aquaporins inhibitors application including Hg^{2+}. They show membrane damage resulting from 500 µM Hg^{2+}. Here we used such a relatively high 500 µM $HgCl_2$ concentration. However, this value was similar to concentrations used in previous studies on whole root systems (Maggio and Joly 1995; Peyrano et al. 1997; Shimizu et al. 2005; Ruggiero et al. 2007). In addition, considering that we worked with sand (in which root excretion of organic compounds created an organic

matrix) and not in hydroponics, the effective concentration in the root zone was probably largely below 500 µM due to immobilization of part of the Hg^{2+} by the system (Ruggiero et al. 2007). The concentration used in this study was chosen from the preliminary dose response curves aimed at identifying a threshold concentration that had a marked effect on sap flow (by instance, 50 µM $HgCl_2$ did not induce any depressive effect on sap flow) but that would not cause apparent irreversible toxicity effects. Indeed, the relation between sap flow and applied pressure was highly linear suggesting that the Hg treatment did not cause broadly deleterious changes in root function during the time course of the pressure flow procedure (data not shown, Maggio and Joly 1995). In addition, there was no significant difference between control and $HgCl_2$-treated roots in the K^+ amount recovered in the xylem exudates delivered through whole-root systems (92.59±9.11%, 122.15±39.63%, 103.30±7.44% and 116.12±19.02% for RIL 043, RIL 127, RIL 149 and RIL 200 in mercury treated plants as compared to the controls, respectively), demonstrating that the Hg^{2+} concentration and exposure durations used here did not poison root cells in a way that might cause them to become leaky to ions (Maggio and Joly 1995). Another convincing argument concerning the lack of general toxicity is the reversal of mercuric chloride inhibition by the scavenger 2-mercaptoethanol which is assumed to remove Hg from membranes of treated roots (Barrowclough et al. 2000). Inhibition of sap flow by $HgCl_2$ was reversed by *ca* 90% following rinsing of the root system by mercaptoethanol solution (10 mM). In addition, sap was spontaneously expressed from cut stems of excised roots several hours after mercury application demonstrating the occurrence of strong root pressure in those roots. Altogether these results indicate that $HgCl_2$ did not reduce sap flow by a general inhibition of root metabolism, but rather by a direct effect on AQPs.

Leaves need to be continuously supplied with water and carbon dioxide to fulfill their photosynthetic function. The water transport capacity of the root (L_0, root hydraulic conductance) is thus a key physiological parameter for whole-plant function since it determines the interplays between sap flow intensity and water potential gradients between soil and leaves. Difference in whole L_0 reached a high value of 60% between sunflower genotypes. L_0 was highest for RIL 149 and lowest for RIL 127 while RIL 043 and RIL 200 had similar intermediate values (Fig. 2A). L_0 reflects the overall water uptake capacity of the root and is attributable to both the root exchange surface (Table 1) and its intrinsic water transport capacity (Lp_r, Fig. 2). Lp_r had a different ranking from L_0, with the highest value for RIL 149 (as was observed for L_0) but the lowest for RIL 043 (whereas it was in second position for L_0). Lp_r in RIL 049 was found to be 70% higher than in RIL 043. Lp_r was

expressed in relation to the whole root surface, assuming that outer cell layers consistently reflected the hydraulic properties of all the segments of the root system (Sutka et al. 2011). We also checked that when Lp_r was expressed in relation to root length or fine root volume, a similar difference between RIL 149 and RIL 043 was observed (see Table 1). Thus, the genotype (RIL 043) can display both high whole root capacity (L_0) and small intrinsic root capacity (Lp_r). Sutka et al. (2011) described a substantial (2-fold) genetic variation in *Lpr*, establishing that *Arabidopsis* root hydraulic properties are far from uniform between natural accessions. We show here that large variations in root hydraulics also appear between sunflower genotypes.

Lp_r was sensitive to brief treatment with $HgCl_2$. This allows Lp_r to be divided into two components: cell to cell and apoplastic pathways (neglecting the dilution-diffusion process across the double layer of membrane lipids which is not sensitive to mercury). The Lp_r of apoplasmic pathways, i.e. Lp_r measured in $HgCl_2$, was identical for all genotypes. In other words, the conductance of the AQP-independent pathway (on an area basis) was similar for all RILs. Tissue mass, organization and/or cell wall structure (suberization of apoplastic barriers usually associated with root maturation which reduces water uptake capacity) may affect intrinsic root hydraulics. However, Sutka et al. (2011) reported that *Arabidopsis* accessions did not show any clear link between root suberization and the hydraulic conductivity of the AQP-independent path. In the present work, although sunflower genotypes displayed evident variation in root anatomy (Table 1), no variation was observed in "intrinsic" apoplastic conductivity (Lp_r in $HgCl_2$ treated roots). This suggests that the cell to cell pathway (aquaporin-dependent path) was the major determinant of the "intrinsic" water transport properties of the organ (Lp_r in control plants). Concerning the contribution of the cell to cell pathway, which is illustrated by "AQPs involvement" (Fig. 3), RIL 149 had the highest involvement of AQPs (72%) while RIL 043 had the lowest (55%). The relative contribution of AQPs to root conductivity (average 60% in the present experiment with sunflower) was similar to other estimates obtained in herbaceous species (Maggio and Joly 1995; Tazawa et al. 1997; Carvajal et al. 1999; Barrowclough et al. 2000; Shimizu et al. 2005; Sutka et al. 2011; Ruggiero et al. 2007) confirming that pathways other than AQPs can make a significant contribution to Lp_r (around 40%).

In our study, sunflower genotypes were selected because of their contrasting water relations under well-watered conditions (Adiredjo et al. 2014). A variety of hydraulic profiles can be observed between the four sunflower genotypes. RIL 149 and RIL 043 had the highest L_0 but exhibited interesting and differing root properties. It appears that "large" root anatomy

(i.e. high root surface, volume, and mass) allows RIL 043 to compensate for its lowest contribution of AQPs to root hydraulics and therefore its lowest Lp_r. Whole L_0 was only slightly lower in RIL 043 than in RIL 149, which had the greater L_0 and the greatest Lp_r due to high AQP involvement. By contrast, RIL 127 had the lowest whole L_0 due to small root development, despite higher intrinsic Lp_r and contribution of AQPs than RIL 043. RIL 200 exhibited intermediate values for all parameters. Interestingly, the ranking of the RILs for L_0 was the same as the ranking of the RILs for WUE of our previous study: RIL 149>RIL 043>RIL 200>RIL 127 (see materials and methods). Therefore, L_0 is suggested to play a key role in sunflower water balance and WUE (Maurel 2007; Sade et al. 2010). AQPs are reported to be regulated by several stresses, particularly drought, and transpiration from shoots (Martre et al. 2001, 2002; Clarkson et al. 2000; Martinez-Ballesta et al. 2002; Shimizu et al. 2005; Sakurai-Ishikawa et al. 2011; Laur and Hacke 2013; Chaumont and Tyerman 2014) often without any change in root anatomy or morphology. Under stress conditions, RIL 043, which displays the highest water transport properties of the whole organ due to high root development, could be less affected than RIL 149 the high transport properties of which depend on its AQP contribution.

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After doing an experiment to evaluate root hydraulic properties in several sunflower genotypes differing in WUE (or CID), the author was interested to better evaluate the correlation of WUE and CID by using the same genotypes of that experiment (Section 4.3). This further experiment (Section 4.4) was not only to analyze the WUE variation v.s root hydraulic properties variations but also to explore the possibility of using CID as an indicator to select sunflower genotypes with high WUE of juvenile sunflowers. As it has already been proved by using two levels of water treatment (well-watered and water-stressed), WUE was strongly and negatively correlated with CID (Section 4.1), in Section 4.4 the author reported the experiment by using five levels of soil water content (SWC) which were maintained stable during the experiments.

4.4 Leaf Carbon Isotope Discrimination as an Accurate Indicator of Water-Use Efficiency in Sunflower Genotypes Subjected to Five Stable Soil Water Contents

Afifuddin Latif Adiredjo[a,b], Olivier Navaud[c], Thierry Lamaze[c*], Philippe Grieu[a*]

[a]Université de Toulouse, INP-ENSAT, UMR 1248 AGIR, BP 32607, 31326 Castanet-Tolosan, France
[b]Brawijaya University, Faculty of Agriculture, Department of Agronomy, Plant Breeding Laboratory, Veteran street, 65145, Malang, Indonesia
[c]Université de Toulouse, UPS, UMR 5126 CESBIO, 18 avenue Edouard Belin, 31401 Toulouse Cedex 9
[*]PhD supervisors

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Correspondence

Philippe Grieu

Université de Toulouse, INP/ENSAT, UMR 1248 AGIR, BP 32607, 31326 Castanet-Tolosan, France
Tel.: +33(0)534323878
Fax.: +33(0)534323901
Email: grieu@ensat.fr

Abstract

Leaf carbon isotope discrimination (CID) has been suggested as an indirect tool for breeding for water use efficiency (WUE) in various crops. This work focused on assessing phenotypic correlations between WUE and leaf CID, and analyzing genotypic variability in four sunflower genotypes grown in a greenhouse in pots with five different stable levels of soil water content (SWC). We measured WUE at whole plant and leaf (intrinsic) level. At whole plant level, WUE was derived from the ratio of total dry aerial biomass (BM) to cumulative water transpired (CWT). At leaf level, intrinsic WUE was calculated as the ratio of light-saturated CO_2 assimilation to stomatal conductance (A/g_s) in younger expanded leaves. Significant differences among the four genotypes and the five SWCs were observed for whole plant and leaf WUE and CID. Strong negative correlations were observed between whole plant WUE and CID as well as between intrinsic WUE and CID with decreasing water availability. No relationships appeared between BM production and WUE or CID. Our results can help agronomists and breeders to evaluate sunflower lines with high WUE for adaptation to drought conditions and for reducing water consumption and crop water needs. Leaf CID appears to be a pertinent and valuable trait to select sunflower genotypes with high WUE.

Keywords: carbon isotope discrimination, water use efficiency, soil water content, sunflower

Introduction

Sunflower (*Helianthus annuus* L.), the fourth important sources of vegetable oil in the world (List 2014), is mainly produced in Ukraine, Russia, European countries, and Argentina (USDA 2014). In recent years, sunflower planted area has increased (Labalette et al. 2012) and expanded in the arid region of the Mediterranean and North Africa (Blamey et al. 1997, Kane et al. 2013). However, in southern Europe it suffers from intense period of water deficit because it is mostly planted in low rainfall areas (Dufresne et al. 2006, Casadebaig et al. 2008). According to Food and Agriculture Organization of the United Nations (FAO) publication reported by Garcia-Vila et al. (2012), sunflower yields vary between <0.5 ton/ha in low rainfall areas and >5 ton/ha under ample water supply. In addition, sunflower is considered well-adapted to drought but genotypes are not homogeneously efficient in the use of water. Systematic analyses of the physiological basis of drought tolerance in sunflower, and purposeful attempts to breed for greater drought resistance are still limited (Grieu et al. 2008).

Water availability is considered to be the main factor limiting ecosystem and agrosystem biomass production. This is because plant growth depends on two closely linked leaf processes, photosynthesis and transpiration. WUE is the ratio between two physiological (transpiration and photosynthesis) or agronomic (yield and crop water use) entities but WUE is mostly discussed in terms of plant production rather than gas exchange (Ehleringer et al. 1993, Ebdon and Kopp 2004). On the one hand, improving WUE would reduce the water requirement for a given yield and thus could help save a considerable amount of irrigation water. On the other hand, an improvement in WUE can significantly increase total biomass production as well as yield at a limited and known soil moisture reserve (Impa et al. 2005). Blum (2009), recently proposed that selection for high WUE in breeding for water limited conditions could lead to reduce yield and drought resistance. However, most of authors argued that the prospect of improving agronomic WUE by breeding for greater WUE has been and remain an attractive challenge (Fischer 1981, Ehleringer et al. 1993, Condon et al. 2004).

Direct measurement of WUE relies either on extensive leaf gas-exchange data or long-term measurements of plant water consumption and biomass production. This is because WUE can be defined either as the ratio of total plant dry matter produced to total water used over the same period or, at leaf level, as the ratio of photosynthetic carbon gain to transpiration water loss (Ehleringer et al. 1993, Condon and Richards 1993, Donovan et al. 2007). These approaches to WUE are logistically difficult in large-scale individual plant screening efforts. It has been

demonstrated, however, that leaf carbon isotope CID can be an excellent surrogate for direct measurement of WUE, and several authors have proposed to use this trait as indirect criterion for yield under drought (Farquhar and Richards 1984, Ehleringer et al. 1993, Condon et al. 2002, Xu et al. 2009).

CID is a measure of the ratio of the stable isotopes of carbon ($^{13}C/^{12}C$) in plant material relative to the value of the same ratio in the atmosphere (Farquhar et al. 1989, Condon 2004). The dominant processes leading to CID are fractionations associated with CO_2 diffusion into leaf intracellular airspaces and with CO_2 carboxylation by the enzyme Rubisco (that catalyzes CO_2 fixation in the Calvin cycle). Discrimination against ^{13}C in leaves during photosynthesis decreases with water stress, mainly because of the lowered stomatal conductance (Farquhar and Lloyd 1993, Ebdon and Kopp 2004). Therefore, CID in plant tissues shows subtle but systematic variations among different plant genotypes and/or species grown under different water conditions (Farquhar and Richards 1984).

The relationships between CID and WUE have been widely explored in several species, especially wheat (including durum wheat) and rice. A negative correlation between CID and WUE in some wheat genotypes was reported by Farquhar and Richards (1984), Misra et al. (2010) and Rizza et al. (2012). Other authors such as Dingkuhn et al. (1991), Scartazza et al. (1998) and Centritto et al. (2009) have also reported a negative correlation between CID and WUE in rice genotypes. However, in sunflower the relationship between CID and WUE has rarely been explored. Lauteri et al. (1993) described a negative correlation between CID and WUE in four sunflower genotypes grown in a greenhouse. In addition, Virgona and Farquhar (1996) and Lambrides et al. (2004) reported the occurrence of correlations between CID and WUE for a range of sunflower genotypes.

In the present study, exploring the possibility of using CID as an indicator to select sunflower genotypes with high WUE, we studied the relationship between CID and WUE in four RILs of juvenile sunflowers. We were particularly interested in evaluating the CID and WUE at five levels of SWC which were maintained stable during the experiments.

Materials and methods

Two experiments were carried out to measure WUE and CID on sunflower plants grown in a greenhouse at the INRA Auzeville station, Toulouse, France (43°31'46,94" N; 1°29'59,71" E). The first experiment (Exp. 1) was done in spring 2012, from 19 March to 1 May 2012 (sowing to

harvest). The second experiment (Exp. 2) was done in autumn, from 17 September to 30 October 2012 (sowing to harvest).

Plant sources

Four recombinant inbred lines (RILs) of sunflower (*Helianthus annuus* L.) from the collection of the Laboratory of Plant-Microbe Interactions (LIPM), INRA Toulouse, France, were used in the two experiments, namely RIL 043, RIL 127, RIL 149 and RIL 200. These four RILs are lines from the INEDI population (Vincourt et al. 2012), which were chosen because of their differing WUE response, determined in a previous experiment (data not shown).

Experimental design and growth conditions

From sowing to harvest, experiments lasted 40 days. Three seeds were sown in each two-liter pot. Ten days after sowing (DAS), the most vigorous plant (based on morphological criteria) in each pot was selected by cutting down the two others. Each pot was put on a scale (maximum capacity 30 kg, precision 2 g, model SXS, GRAM, Spain) connected by interface wireless communication to a computer with installed software (ENSAT 1.07T, developed by Pesage du Sud Ouest, Launaguet, France).

Starting at 21 DAS, the plants were subjected to different water treatments. Soil water conditions were maintained by daily weighing of the pots and watering on the basis of weight loss (the increase in plant weight was considered negligible).

The experiments were arranged in a randomized complete block design with four RILs, five water treatments and five replicates.

Water treatments and greenhouse conditions of experiment 1 (19 March – 1 May 2012)

In Exp. 1, water treatments were applied consisting in five levels of SWC: 35%, 23%, 21%, 18% and 16%. Pots contained 2 kg of a mixture of soil collected from the field (50%), organic matter (30%) and sand (20%). SWC was determined by the gravimetric method described by Lambe and Whitman (1969).

The trials were carried out under well-controlled conditions. Air temperature (T) and relative humidity (RH) were automatically recorded every 30 minutes. Air vapor pressure deficits (VPD) were calculated as described by Allen et al. (1998): $VPD = es - ea$; $es = 0.6108 \times \exp[17.27T/(T + 237.3)]$; $ea = es \times (RH/100)$, where es is the saturation vapor pressure (kPa), T, the mean air temperature (°C), RH, the relative humidity of the air (%).

During the photoperiod (from 05:30 to 18:30 CET), the air temperatures were: minimum (Tmin) 16.7^{0}C; maximum (Tmax) 23.6^{0}C and mean (Tme) 20.8^{0}C. The relative humidity was: minimum (RHmin) 29.4%; maximum (RHmax) 52.3% and mean (RHme) 36.6%. The vapor pressure deficits (Fig. 1A) were: minimum (VPDmin) 1.80 kPa, maximum (VPDmax) 4.40 kPa and mean (VPDme) 2.81 kPa.

Water treatments and greenhouse conditions of experiment 2 (17 September – 30 October 2012)

In Exp. 2, water treatments consisted of five levels of SWC: 25%, 20%, 16%, 13% and 10%. Pots were filled with soil extracted from the field and sand in equal proportions.

During the photoperiod (from 05:30 to 17:30 CET), the following parameters were measured: Tmin 17.8^{0}C, Tmax 26.2^{0}C and Tme 23^{0}C; RHmin 31.3%, RHmax 61.7% and RHme 48.8%; VPDmin 1.14 kPa, VPD max 2.26 kPa and VPDme 1.61 kPa (Fig. 1A).

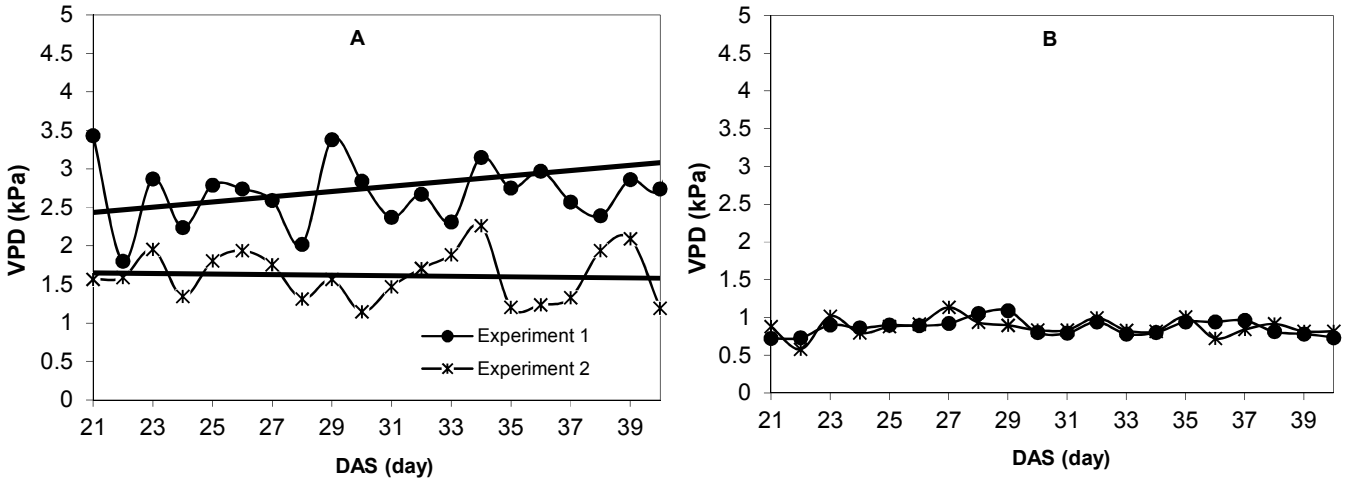

Fig. 1 Vapour pressure deficit (VPD) in the day (A) and in the night (B) in greenhouse during the Exp.1 and Exp.2. The data represent the mean of VPD in the day (during photoperiod) and in the night. The linear lines in figure A represent the average of VPD in the Exp. 1 and Exp. 2. DAS: days after sowing.

Trait measurements

Agronomic traits and water use efficiency

At the end of the experiments (23 DAE), the above-ground parts of the plants were harvested. Stems and leaves were oven dried at 80^{0}C for 48h until they reached constant mass to determine total dry aerial biomass (BM).

Transpiration water loss (WT) for each plant was estimated every day from the difference in the pot weight. Total transpiration (cumulative water transpired, CWT) for each plant was determined at the end of the experiment by accumulating daily WT. WUE (on a whole plant basis) was determined at the end of the experiments as the ratio of BM to CWT.

Leaf gas exchange measurements and intrinsic water use efficiency (A/g_s)

Measurements of CO_2 assimilation rates under saturating light (A) and stomatal conductance (g_s) were made with a portable Li-6400 (Li-Cor, Lincoln, NE, USA) between 09:00 and 12:00 (Central European Time) in Exp. 2 (from 19 to 21 DAE). All the measurements were made on a fully-expanded leaf (one per plant) under 1500 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and 40 Pa CO_2 partial pressure. Leaf temperature was maintained at 25 ± 2^0C and RH was 50%.

Carbon isotope discrimination

Oven-dried leaves (including petioles) of each plant were ground into a homogeneous fine powder and 2-3 mg subsamples were weighed and placed in capsules (Elemental Microanalysis, Okehampton UK) to be analyzed using a continuous flow Isotope Ratio Mass Spectrometer (Sercon Ltd., Cheshire, UK) at UC Davis Stable Isotope Facility (California, Davis, USA). Carbon isotope composition (δ) was calculated relative to the international Pee Dee Belemnite (PDB) standard (Farquhar et al. 1989): $\delta_{plant} = (R_{sa} - R_{sd})/R_{sd}$ X 1000 [‰] where R_{sa} and R_{sd} are the ^{13}C : ^{12}C ratios of the sample and the standard, respectively (Craig 1957). Carbon isotope discrimination (CID) was estimated as: $CID = (\delta_{air} - \delta_{plant})/(1 + \delta_{plant}/1000)$ where δ_{air} is the ^{13}C composition of atmospheric CO_2, which is assumed to be –8.0‰ (Farquhar et al. 1989).

Statistical analysis

Data were tested for normal distribution with the Kolmogorov-Smirnov test. All statistical analysis was done using the statistical package PASW statistics 18 (IBM, New York, USA). Analysis of variance (ANOVA) was used to calculate the effects of genotypes and SWC. For each ANOVA, a trait was considered as a dependent variable. Genotype, SWC and replicate were considered as the fixed factors. Means were compared using a Student-Newman-Keuls (SNK) test ($P < 0.05$). Pearson's correlation coefficients were calculated to determine the phenotypic

relationships between WUE, CID and related traits (BM, CWT). Coefficient of determination (R^2) was calculated by determining the regressions of main traits, CID and WUE.

Results

Relationships between water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT)

In the two experiments, a highly significant negative correlation was observed between WUE and CID (Table 1, r_p = -0.66, $P < 0.001$, n = 20 in Exp. 1, and r_p = -0.67, $P < 0.001$, n = 20 in Exp. 2) while there were no significant correlations between BM and CID or WUE. In contrast, there was a significant negative correlation between WUE and CWT but only in Exp. 2 (r_p = -0.55, $P < 0.01$, n = 20). In the two experiments, the coefficient of determination between WUE and CID was high (0.79 in Exp. 1 and 0.81 in Exp.2; Fig. 2). In the two experiments, there was a concomitant increase in WUE and a decrease in CID from the high to the low SWC for all genotypes. Thus, the highest values of WUE and the smallest values of CID were observed at the smallest SWC, whereas the smallest values of WUE and the highest values of CID were observed at the highest SWC (Fig. 3).

Table 1 Phenotypic correlations (r_p) between water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) for four RILs and five soil water contents (n = 20, average of five replicates)

Traits	WUE (g.kg-^{1})	CID (‰)	BM (g)
Experiment 1			
CID (‰)	-0.66***		
BM (g)	-0.09 ^{ns}	0.44 ^{ns}	
CWT (ml)	-0.37 ^{ns}	0.62***	0.92***
Experiment 2			
CID (‰)	-0.67***		
BM (g)	0.39^{ns}	0.18 ^{ns}	
CWT (ml)	-0.55**	0.81***	0.50**

** Significant at $P < 0.01$, *** Significant at $P < 0.001$, ^{ns} Not significant

Genotypic variability in water use efficiency (WUE) and carbon isotope discrimination (CID) in plants growing on five stable soil water contents (SWC)

Mean values of WUE were lower in Exp.1 than in Exp. 2 (1.58 g.kg^{-1} and 2.03 g.kg^{-1}, respectively) whereas mean values of CID were higher in Exp. 1 than in Exp. 2 (23.45‰ and 22.37‰, respectively). During Exp. 1, WUE values ranged from 0.55 to 3.13 g.kg^{-1} and CID

values ranged from 21.50 to 24.88‰ (Table 2). The variances of WUE and CID were 0.34 and 0.71, respectively. During Exp. 2, WUE values ranged from 0.79 to 4.32 $g.kg^{-1}$ and CID values ranged from 21.50 to 24.88‰. The variances of WUE and CID were 0.54 and 2.27, respectively. These results showed a narrower genotypic variability for WUE and CID in Exp. 1 than in Exp. 2. ANOVA results showed that there were significant effects of genotype and SWC for WUE and CID in the two experiments. Moreover, there was no significant effect of the genotype and SWC interaction for these two traits in the two experiments.

In Exp. 1, there were no significant differences between genotypes for WUE except for RIL 200 at 16% SWC (Fig. 3A). WUE values were very low (the power of ANOVA was 0.77; data not shown). In contrast, for CID, significant differences between genotypes appeared at all five SWCs (Fig. 3C). This is consistent with the results for CID of Exp. 2 where genotypes showed differences, with the same ranking as in Exp.1, under all five SWCs (Fig. 3D). In Exp. 2, unlike in Exp.1, significant differences were obtained in WUE between genotypes for all SWCs (Fig. 3B).

Leaf gas exchange, intrinsic water use efficiency (A/g_s) and carbon isotope discrimination (CID) in experiment 2

Measurements of gas exchange for the five stable SWCs in Exp. 2 showed a decrease in A (light-saturated CO_2 assimilation) and g_s (stomatal conductance) as water availability decreased. Therefore, high values for A and g_s, (27 μmol $CO_2.m^{-2}.s^{-1}$ and 0.68 mol $H_2O.m^{-2}.s^{-1}$, respectively) were observed at the highest SWC (25%) whereas low values of A and g_s were reached (1.70 μmol $CO_2.m^{-2}.s^{-1}$ and 0.02 mol $H_2O.m^{-2}.s^{-1}$, respectively) at the smallest SWC level (10%, Table 3). The values of intrinsic water use efficiency (A/g_s) ranged from 24.29 μmol $CO_2.mol^{-1}$ H_2O to 136.36 μmol $CO_2.mol^{-1}$ H_2O.

ANOVA showed that A was not significantly different between genotypes but that significant differences appeared for CO_2 assimilation between SWC levels. By contrast, g_s and A/g_s were significantly different both between genotypes and SWC (Table 3).

Positive correlations were observed between CID and all leaf gas exchange traits (Table 4). A small but very significant phenotypic correlation was obtained between CID and A (r_p = 0.47, $P < 0.001$, n = 100) as well as between CID and g_s (r_p = 0.45, $P < 0.001$, n = 100). CID and A/g_s were negatively correlated (r_p = -0.30, $P < 0.001$, n = 100) and A/g_s was negatively correlated with A and g_s (r_p = -0.47, $P < 0.001$, n = 100 for A/g_s and A; r_p = -0.72, $P < 0.001$, n = 100 for A/g_s and g_s).

Table 2 Genotypic variation, the mean squares of analysis of variance (MS ANOVA) for water use efficiency (WUE), carbon isotope discrimination (CID), biomass (BM) and cumulative water transpired (CWT) among four RILs, five soil water contents (SWC) and five replicates in Exp. 1 and Exp. 2 (n = 100 for each experiment)

Trait	Minimum	Maximum	Mean	Std. deviation	Variance	MS ANOVA		
						Genotype	SWC	Genotype x SWC[a]
Experiment 1								
WUE (g.kg-^{1})	0.55	3.13	1.58	0.86	0.34	0.94*	1.35***	0.35 ^{ns}
CID (‰)	21.50	24.88	23.45	0.84	0.71	4.31***	6.19***	0.27 ^{ns}
BM (g)	0.07	1.87	0.58	0.36	0.13	0.27^{ns}	0.59***	0.06 ^{ns}
CWT (ml)	121.00	991.00	387.39	180.42	32552.38	27573.21^{ns}	438588.76***	13247.16 ^{ns}
Experiment 2								
WUE (g.kg-^{1})	0.79	4.32	2.03	0.72	0.51	9.14***	0.85**	0.30 ^{ns}
CID (‰)	19.68	25.47	22.37	1.51	2.27	12.54***	22.43***	1.61 ^{ns}
BM (g)	0.21	1.06	0.50	0.19	0.03	0.33***	0.34***	0.02 ^{ns}
CWT (ml)	105.00	515.00	264.00	95.80	9178.21	26889.10***	156976.81***	3264.78 ^{ns}

* Significant at $P < 0.05$, ** Significant at $P < 0.01$, *** Significant at $P < 0.001$, ^{ns} Not significant, [a] Genotype and SWC interaction.

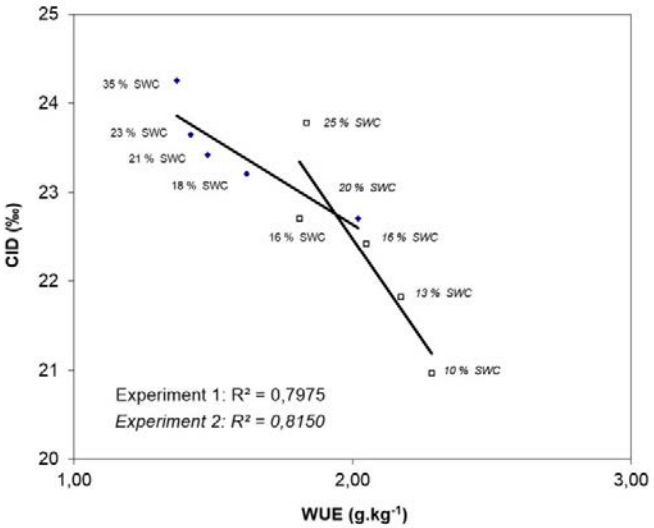

Fig. 2 Relationships between water use efficiency (WUE) and carbon isotope discrimination (CID) in five soil water contents (SWC) for the Exp. 1 and Exp. 2. For each experiment, values represent mean of four RILs and five replicates (n = 5).

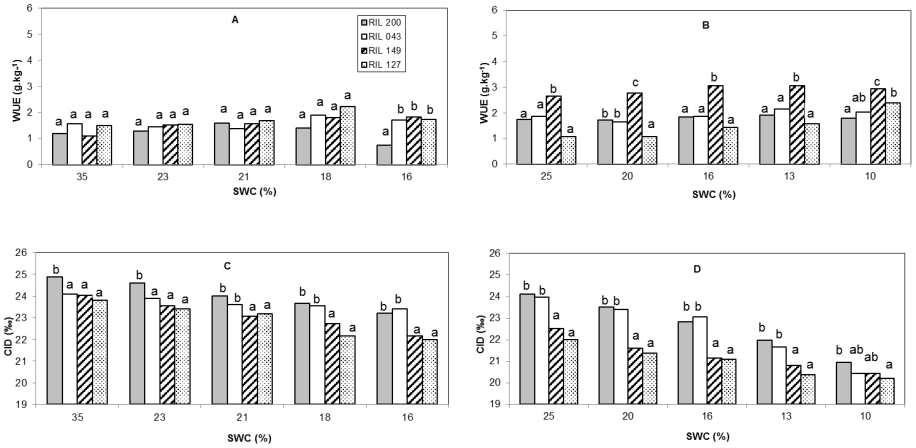

Fig. 3 Water use efficiency in Exp. 1 and Exp. 2 (A and B), and carbon isotope discrimination in Exp. 1 and Exp. 2 (C and D), subjected to five soil water contents (SWC) of four genotypes (RIL 200, RIL 043, RIL 149, RIL 127). Different letters in each SWC level represent significant differences among genotypes (SNK's test, $P < 0.05$).

Table 3 Genotypic variation of net CO_2 assimilation rates (A), stomatal conductance (g_s), intrinsic water use efficiency (A/g_s) among four RILs, five soil water contents (SWC) and five replicates in Exp. 2 (n = 100)

Trait	Minimum	Maximum	Mean	Std.deviation	Variance	Mean square	
						Genotype	Soil water content
A (μmol $CO_2.m^{-2}.s^{-1}$)	1.70	27	16.68	6.59	43.37	13.53^{ns}	698.12***
g_s (mol $H_2O.m^{-2}.s^{-1}$)	0.02	0.68	0.33	0.18	0.03	0.04*	0.58***
A/g_s (μmol $CO_2.mol^{-1}$ H_2O)	24.29	136.36	59.57	23.04	530.88	1304.38**	5915.00***

* Significant at $P < 0.05$, ** Significant at $P < 0.01$, *** Significant at $P < 0.001$, ^{ns} Not significant

Table 4 Phenotypic correlations (r_p) between carbon isotope discrimination (CID), net CO_2 assimilation rates (A), stomatal conductance (g_s), intrinsic water use efficiency intrinsic (A/g_s) among four RILs, five soil water contents (SWC) and five replicates in Exp.2 (n = 100)

Trait	CID ‰	A μmol CO_2. $m^{-2}.s^{-1}$	g_s mol H_2O. $m^{-2}.s^{-1}$
A (μmol $CO_2.m^{-2}.s^{-1}$)	0.47***		
g_s (mol $H_2O.m^{-2}.s^{-1}$)	0.45***	0.90***	
A/g_s (μmol $CO_2.mol^{-1}$ H_2O)	-0.30***	-0.47***	-0.72***

*** Significant at $P < 0.001$

Discussion

In this study we used five levels of soil moisture, which were maintained rigorously constant throughout the duration of the experiments. This is the first report to our knowledge of such stabilized treatments being used to study the effect of water limitation on sunflower grown for several weeks in a greenhouse. The levels of SWC defined here covered a large gradient of water availability, leading to differing plant physiological behavior. This is demonstrated by the marked differences observed in the rates of CO_2 assimilation and values of stomatal conductance between plants grown at the highest or the lowest soil moisture. Changes in SWC led also to changes in whole plant WUE (BM/CWT) and intrinsic leaf WUE (A/g_s), and in leaf CID. WUE and A/g_s were strongly and negatively correlated with CID. This is in accordance with previous work (Lauteri et al. 1993, Lambrides et al. 2004) and agrees with the model of Farquhar and Richards (1984) developed for wheat. WUE in Exp.1 was lower than in Exp. 2. This can be explained by the differences in average VPD values in the greenhouse during the two experiments (Fig. 1A) since VPD was higher in Exp. 1 than in Exp. 2.

WUE has often been shown to be related to biomass production in plants. The relationship can be positive or negative (Li 1999). In sunflower, a positive correlation between WUE and BM was found by Virgona and Farquhar (1996) and Lambrides et al. (2004). If WUE and BM are positively correlated, plants that use water more efficiently by producing greater biomass for a given quantity of water transpired would also grow more rapidly and produce higher BM (Wright et al. 1993). In the present study with sunflower, no correlation (positive or negative) was found between the two parameters (Table 1). This agrees with the observation of Misra et al. (2010) on 20 durum wheat genotypes. Thus, a plant which displayed high WUE may not produce higher BM. This may be because higher WUE is generally achieved by plant traits than lower transpiration (such as reduced leaf area, moderate growth and low stomatal conductance) reducing photosynthesis and therefore yield.

Variations in WUE are mainly due to leaf diffusive characteristics (such as stomatal conductance, g_s) and intrinsic photosynthetic capacity (such as Rubisco capacity). Since BM production is closely associated with transpiration, in plants where WUE is principally determined by intrinsic leaf photosynthetic capacity ("capacity type plant"), WUE is weakly dependent on transpiration, and high WUE may be associated with high biomass production. Plants that maximize WUE through a reduction in transpiration (gs) are called "conductance type" (Farquhar and Lloyd 1993, Impa et al. 2005). Reduction of transpiration to increase WUE

often results in reduced crop yield potential under most dryland situations, where crops depend on unpredictable seasonal rainfall rather than a limited and known soil moisture reserve. However, in "conductance type" plants, higher WUE must lead to higher BM production for a given and limited amount of transpirable soil water. In the present study, WUE was negatively correlated with CWT (Exp. 2). In addition, the absence of relationships between WUE and BM production may be because stable soil moisture (i.e. constant water amount) was maintained in the pot that did not correspond to a limited transpirable soil water reserve.

Leaf CID decreased in sunflower plants grown with decreasing SWC ($R^2 = 0.79$ for Exp. 1; $R^2 = 0.81$ for Exp. 2, Fig. 2). This trend of decreasing CID with decreasing water availability has been reported in previous studies, for example in wheat (Farquhar and Richards 1984), barley (Hubick and Farquhar 1989), Russian wild rye (Frank and Berdahl 2001), and rice (Zhao et al. 2004). However, most of these authors used only two water regimes for the plants (well-watered and markedly water-stressed conditions). Indeed, studies that report CID values for more than two levels of water are scarce: Zhao et al. (2004) found a negative relationship between CID and WUE of two upland rice cultivars under three water regimes and Erice et al. (2011) found a negative correlation in four alfalfa genotypes subjected to progressive drought. In the present approach, graduated water limitation was imposed on plants through five levels of soil moisture. Often in the literature, soil moisture is not well controlled and it can vary, especially at the beginning of the experiments. In our study, SWC was precisely monitored daily so that leaf CID and WUE values of sunflower plants presented in the present work are related to accurate levels of SWC.

The strong correlation between CID and WUE among sunflower RILs indicates that the relationships may be used to select sunflower varieties with high WUE through leaf CID. Significant differences among four RILs and five SWC were observed for CID and WUE. Such differences in CID and WUE among genotypes have been reported by many authors, for example in rice (Zhao et al. 2004), alfalfa (Erice et al. 2011), and *Eucalyptus microtheca* F. Muell. (Li 1999). In our study, two contrasting RILs were observed: RIL 149, identified as having low CID and high WUE, and RIL 200, identified as having high CID and low WUE. The consistency of CID ranking and its strong negative relationship with WUE in two experiments carried out in different seasons, VPD, SWC and on different types of soil reinforce the possibility of using this trait as a pertinent tool for agronomists and breeders in order to select sunflower genotypes with high WUE. Irrigated agriculture represents up to 85% of total human water consumption. Thus,

considering world population expansion, it is imperative to improve WUE of irrigated but also of rain-fed crops (This et al. 2010).

The wide range observed in this study for CID in Exp. 1 (absolute value of 3.38‰, from 21.50 to 24.88‰) and in Exp. 2 (5.79‰, from 19.68 to 25.47‰), exceeds the range of 2.8‰ reported by Lauteri et al. (1993) on sunflowers grown in a greenhouse. Lambrides et al. (2004) found variations of 4.4‰ (absolute units) for 161 sunflower genotypes grown in field conditions. The CID ranges found in the present study are in agreement with these authors. In addition, in previous experiments on a larger number of sunflower genotypes (150 RILs), we observed ranges of 8.95‰, 5.82‰ and 6.91‰ in 2010, 2011 and 2012, respectively (unpublished data). Such wide ranges of CID suggest that it could possibly be used as a selection criterion in sunflower breeding programs. Due to the wide range of CID, using this trait rather than WUE might be more suitable for comparing genotypes subjected to drought.

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5 GENERAL CONCLUSION AND PERSPECTIVES

The results and discussion of this Thesis have been structured in four sections (Chapter 4) corresponding to its publications. The relevance of the findings is extensively discussed in each of these Sections that deal with each specific objective. Therefore, the present General Conclusions and Perspectives chapter focuses only on broad and integrated view of WUE and CID in sunflower that deals with general objectives.

5.1 General conclusion

5.1.1 Can WUE be determined by using CID on sunflower?

WUE measurement is logistically difficult in large-scale individual plant screening efforts. However, the difficulty has been overcome since the WUE has been found to be negatively associated with CID (Farquhar et al. 1982; Farquhar and Richards, 1984). In the present Thesis, the significant negative correlations were observed between WUE and CID in all experiments across different water regime (Section 4.1 and 4.4). These results are in accordance with previous work in sunflower (Lauteri et al. 1993; Lambrides et al. 2004) and agree with the model of Farquhar and his colleagues developed for wheat. Significant correlations between WUE and CID indicated that the use of CID could be a useful tool for analyzing genetic variation of WUE within sunflower genotypes, which has also been shown in other crops, for example in peanut (Hubick et al. 1986), tomato (Martin et al. 1989), rice (Scartazza et al. 1998), *Eucalyptus microtheca* F. Muell. (Li, 1999), barley (Anyia et al. 2007), and sugar beet (Rajabi et al. 2009). The strong correlations found between WUE and CID highlight the potential of CID to be an excellent surrogate for direct measurement of WUE in sunflower.

In addition, the WUE and CID in the present Thesis have been determined on different scenario of drought (Section 4.1) and on five levels of water regime (Section 4.4) which are different with other previous studies in sunflower that carried out only in a well-watered condition (Lambrides et al. 2004) or in two levels of water regime with only one level in a drought condition (Lauteri et al. 1993). The different drought condition of examining relationship between WUE and CID give robustness to the results. Moreover, by using CID rather than WUE, it will enable agronomists and breeders to distinguish the genotype under drought condition, and therefore CID is

recommended as a screening tool for WUE under the experimental conditions of the present Thesis.

5.1.2 How WUE and CID variations analysis can contribute to the genotypic selection of sunflower subjected to drought?

In two experiments (Exp. 2011 and Exp. 2012), by using 150 genotypes, a similar range of values for WUE and CID was observed, despite their drought scenario differed (Section 4.1). That was likely because the genotypes were a RILs population which had been constructed from parents with specific response to water use (Rengel et al. 2012). In Exp. 2011 and Exp. 2012, XRQ identified as having either low WUE or high CID while PSC8 identified as having either high WUE or low CID (Appendix 2). This consistent phenotypic data coupled with the positive rank correlation across two experiments for both WUE and CID. However, the correlation coefficient for WUE was smaller than the correlation coefficient for CID. This result indicated that CID is more stable than WUE across different drought conditions. The stability of CID was also observed between the water regimes (WW and WS conditions) (Appendix 3). Additionally, the wide range observed for CID in Exp. 2011 (5.82‰) and in Exp. 2012 (6.91‰), exceeds the range of 4.4‰ reported by Lambrides et al. (2004) on 161 sunflower genotypes grown in field conditions, as well as the wide range observed for CID in the experiment by using four RILs (3.38‰ and 5.79‰ for experiment 1 and 2, respectively), exceeds the range of 2.8‰ reported by Lauteri et al. (1993) on some sunflower genotypes grown in a greenhouse. In summary, stability of CID values across different environmental conditions and wide ranges of CID suggest that using this trait rather than WUE might be more suitable for comparing genotypes subjected to drought, and therefore, CID may be a very useful substitute for improving WUE of sunflower in genotypic selection program (breeding).

Results of QTL mapping in the present Thesis, which is the first report of QTL for WUE and CID in sunflower subjected to drought, showed that nine QTL controlling WUE and eight controlling CID were identified across the two experiments. From these QTL, some QTL were located on the same chromosome or on a similar QTL position (co-localization). The occurrence of QTL for WUE associated with QTL of CID at the same locus may be explained by the fact that (i) the QTL are closely linked genetically or (ii) a single locus controls multiple traits and a gene may have pleiotropic effects (Li et al. 1995; Laza et al. 2006). Nevertheless, only the QTL for CID showed the stability of their effects across different water regimes, since the three QTL

for CID of the three different water regimes were detected on the same chromosome (on chromosome LG13). Therefore QTL for CID can be considered as a "constitutive" QTL (Collins et al. 2008). The QTL mapping for WUE and CID in the present Thesis confirmed the polygenic inheritance of CID in sunflower by detecting multiple QTL controlling CID. Finally, the results indicated that CID can be used to improve WUE by using marker-assisted selection (MAS) approaches, especially in creating sunflower genotypes: not only with improved drought tolerance but also with high productivity, and therefore help to maintain the stability of sunflower crop production.

5.1.3 Can WUE variation be revealed by the variation of plant-water relation traits, i.e. control of transpiration, water extraction capacity, dehydration tolerance and root hydraulic conductance?

In the present Thesis, variation of the plant-water relation traits including FTSWt (control of transpiration), TTSW (water extraction capacity) and OA (dehydration tolerance) was analyzed by QTL mapping. Results of QTL mapping for control of transpiration and water extraction capacity traits are extremely valuable in this Thesis because QTL for FTSWt in crops and QTL for TTSW in sunflower have never been reported in the literature. A QTL for FTSWt co-localized with QTL for TTSW. Further, the genetic control between FTSWt and OA, as well as between TTSW and OA was completely independent, as no co-localization of QTL was observed. This was consistent with the phenotypic data that no significant correlation was found among the traits except between TTSW and osmotic potential (but weak correlation). In addition, the QTL of FTSWt and TTSW was identified on chromosome LG14 and LG06 (only a QTL of TTSW) where the QTL for WUE, CID and biomass were also identified on those chromosomes. These findings suggested that the genetic control of FTSWt and TTSW was dependently and genetically linked with WUE, CID and biomass rather than with OA. Therefore, detailed characterization of these genomic regions will lead to an improved understanding of drought tolerance and might set the stage for the positional cloning of drought tolerance genes.

Variation of root hydraulic conductance (L_0) and/or root conductivity (Lp_r) in the present Thesis is not analyzed by QTL mapping because these traits were only measured on four selected RILs (Section 4.3). A variation of hydraulic profiles can be observed between the four RILs. RIL 043 and RIL 149 had the highest L_0 but exhibited differing root properties. It appears that "large" root anatomy (i.e. high root surface, volume and mass) allows RIL 043 to compensate for its lowest

contribution of AQPs to Lp_r and therefore its lowest root intrinsic conductivity (Lp_r). L_0 was only slightly lower in RIL 043 than in RIL 149, which had the greater L_0 and the greatest Lp_r due to high AQPs involvement. Interestingly, RIL 149 of the control plants exhibited the greatest value for L_0 where this RIL has also been identified as having high WUE (Section 4.4). In contrast, RIL 200 which exhibited intermediate value of L_0 has been identified as having low WUE. Therefore, L_0 is suggested to play a key role in sunflower water balance and WUE variation and is agreement with other previous authors (Maurel, 2007; Sade et al. 2010). In addition, this work is a preliminary approach to study L_0 and Lp_r in sunflower.

5.2 Perspectives

More research is needed to further dissect the major QTL region for low CID by using other sunflower populations and different environmental conditions, as well as other drought scenarios to understand the complex of genetic mechanisms underlying this constitutive trait. Thus, the robustness of the results from the present Thesis could be examined. Despite QTL mapping is useful for enhancing the target trait using genetic transformation, this method is only the first step to genomic technologies. The ultimate goal is to tag and isolate genes or beneficial QTL alleles controlling WUE and CID. With the application of marker assisted selection (MAS), favorable alleles can be introduced into elite germplasm to derive improved cultivars and accelerate plant breeding process in water-limited environments.

Results of QTL mapping are needed to be explored by other disciplines such as eco-physiological or crop growth modelling, because eco-physiological modelling has so far contributed little to QTL analysis of a quantitative trait. Therefore, the ability of eco-physiological model to assist QTL analysis by using WUE or CID and other related traits including FTSWt, TTSW and OA is required to be examined. In addition, this study highlights the importance of studying the WUE in sunflower by combining the physiological and genetic analysis.

The author has presented and discussed all the findings that deals with specific and/or general objectives, and has provided the concluding remarks to respond the three general objectives. However, all information in the present Thesis focuses only on: (i) one population, i.e. 150 RILs of the INEDI population, (ii) greenhouse experiments, and (iii) juvenile sunflower. Therefore, the extensive research is seemly required to be done: (i) by using other sunflower populations, (ii) by

conducting the experiment in field conditions with different crop management, and (iii) by determining WUE in several growth stages of sunflower (particularly after anthesis).

Lastly, several traits that were analysed in the present Thesis, especially plant-water relation traits, such as FTSWt and TTSW, have been identified able to reveal the genetic control of WUE and CID. However, other experiments by using a large number of genotypes, mainly by using other populations, are needed to analyse the genotypic variability of FTSWt and TTSW in order to generate drought tolerance sunflower.

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APPENDICES

Appendix 1 List of recombinant inbred lines (RILs) that used in the experiments of the present Thesis.

Number	Genotype	Number	Genotypes	Number	Genotypes	Number	Genotypes
1	RIL001	31	RIL072	81	RIL174	131	RIL006
2	RIL002	32	RIL077	82	RIL175	132	RIL009
3	RIL004	33	RIL078	83	RIL177	133	RIL012
4	RIL005	34	RIL084	84	RIL178	134	RIL013
5	RIL007	35	RIL085	85	RIL179	135	RIL016
6	RIL008	36	RIL088	86	RIL181	136	RIL019
7	RIL010	37	RIL089	87	RIL183	137	RIL020
8	RIL011	38	RIL090	88	RIL184	138	RIL021
9	RIL014	39	RIL098	89	RIL185	139	RIL022
10	RIL015	40	RIL103	90	RIL186	140	RIL024
11	RIL051	41	RIL104	91	RIL187	141	RIL025
12	RIL026	42	RIL105	92	RIL189	142	RIL027
13	RIL030	43	RIL107	93	RIL190	143	RIL028
14	RIL031	44	RIL110	94	RIL057	144	RIL063
15	RIL032	45	RIL111	95	RIL192	145	RIL034
16	RIL033	46	RIL115	96	RIL193	146	RIL058
17	RIL035	47	RIL117	97	RIL194	147	RIL039
18	RIL037	48	RIL119	98	RIL195	148	RIL046
19	RIL040	49	RIL125	99	RIL196	149	RIL048
20	RIL041	50	RIL127	100	RIL197	150	RIL060
21	RIL042	51	RIL128	101	RIL198		
22	RIL043	52	RIL129	102	RIL199		
23	RIL044	53	RIL130	103	RIL200		
24	RIL045	54	RIL136	104	RIL201		
25	RIL047	55	RIL137	105	RIL202		
26	RIL052	56	RIL138	106	RIL208		
27	RIL056	57	RIL140	107	RIL209		
28	RIL059	58	RIL141	108	RIL210		
29	RIL062	59	RIL142	109	RIL212		
30	RIL071	60	RIL143	110	RIL214		
31	RIL072	61	RIL144	111	RIL215		
32	RIL077	62	RIL054	112	RIL216		
33	RIL078	63	RIL146	113	RIL217		
34	RIL084	64	RIL148	114	RIL231		
35	RIL085	65	RIL149	115	RIL235		
36	RIL088	66	RIL151	116	RIL238		
37	RIL089	67	RIL152	117	RIL240		
38	RIL090	68	RIL153	118	RIL241		
39	RIL098	69	RIL155	119	RIL248		
40	RIL103	70	RIL156	120	RIL251		
41	RIL104	71	RIL158	121	RIL260		
42	RIL105	72	RIL160	122	RIL262		
43	RIL107	73	RIL161	123	RIL263		
44	RIL110	74	RIL163	124	RIL269		
45	RIL111	75	RIL166	125	RIL270		
46	RIL115	76	RIL167	126	RIL272		
47	RIL117	77	RIL168	127	RIL275		
48	RIL119	78	RIL169	128	RIL278		
49	RIL125	79	RIL170	129	XRQ		
50	RIL127	80	RIL171	130	PSC8		

Appendix 2 Phenotypic data of WUE and CID for XRQ and PSC8 in two experiments (Exp. 2011 and 2012) under well-watered (WW) and water-stressed (WS) conditions. Data represent mean of the replicates of 150 RILs (n = 150).

Genotype	WUE_{T2011} (g/kg)		WUE_{E2011} (g/kg)		WUE_{T2012} (g/kg)		CID_{2011} (g/kg)		CID_{2012} (g/kg)	
	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS
XRQ	2.98	3.02	1.49	1.8	2.32	2.41	25.8	23	25.5	22.8
PSC8	3.33	3.34	2.29	2.59	3.36	3.47	25.6	22.8	24.5	22.7

Appendix 3 Relationship between (A, B, C) WUE and (B) CID values for 150 recombinant inbred lines (RILs) determined in two experiments (Exp. 2011 and 2012): (A) WUET2011, (B) WUEE2011, (C) WUET2012, (D) CID in Exp. 2011, (E) CID in Exp. 2012. Data represent mean of the replicates of 150 RILs (n = 150).