Identification and characterization of a tomato introgression line with reduced wilting under drought

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Statement of authorship

This thesis is an account of research undertaken between February 2007 and December 2010 at the Research School of Biology, The Australian National University, Canberra, Australia. Except where acknowledged in the customary manner, the material presented in this thesis is, to the best of my knowledge and belief, original and has not been submitted in whole or in part for a degree in any other university or institution of higher learning. This thesis comprises a general introduction, three chapters of results and a conclusion chapter. The contributions of myself and others to each section are described below.

Dr. L. E. P. Peres (University of São Paulo) performed the initial crosses, provided seeds of Micro-Tom and WELL and of the MT sp^{+}/sp^{+} line. Dr Peres also designed and performed the initial phenotypic screens of the MicroTom × *S. pennellii* progeny described in Chapter 2.

All other experiments were conducted at the Research School of Biology, ANU, under the joint supervision of Dr Josette Masle and Dr. David Jones, who contributed to experimental design, data analysis and interpretation, and edited drafts of all chapters.

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Summary

Population growth and climate change pose a serious challenge to food supply. Agriculture is the biggest consumer of freshwater in the world. With widespread water scarcity and expected changes in rainfall patterns, both boosting plant yield using the same amount of water and increasing the survival and yield of crops under drought are top priorites for plant biologists. The understanding of genetic and physiological mechanisms controlling water-use efficiency (WUE) and of plant resistance to drought is, however, still limited. Tomato (*Solanum lycopersicum* L.) is an excellent genetic model with a rich source of natural variation in its wild relatives. *S. pennellii* Correll, among them, is adapted to the arid conditions of the Andean region in South America and exhibits a high tolerance to drought and increased WUE, measured as biomass gained per unit of water lost. In this work, a series of crosses and screening steps were done with the aim of introducing some of the genetic determinant(s) of *S. pennellii*'s adaptation to drought into cultivated tomato (miniature cultivar Micro-Tom). Selecting hybrids with delayed wilting, a homozygous line was found which showed delayed wilting upon water deprivation and increased WUE. This novel genotype, named WELL (an acronym for Water Economy Locus in Lycopersicon) exhibited pleiotropic traits, including semi-determinate growth habit, elongated internodes, and more erect, wrinkled leaves. The introgressed segment was mapped to a pericentromeric region of 42 to 54 cM on the long arm of chromosome *1*, which comprises the *yellow* fruit epidermis pigmentation gene. Physiological analyses showed that WELL leaves have lower stomatal conductance than their Micro-Tom counterparts under drought, in spite of a similar or slightly increased stomatal density, implying more closed stomata

i.e. an increased stomatal sensitivity to water deprivation in WELL leaves. Recombinant lines with reduced introgressions (1-24 cM) were generated. Their preliminary analysis indicated that some of the pleiotropic traits in WELL were not genetically linked to the delayed wilting phenotype and two of the recombinant lines appeared to have altered growth responses under drought, but this deserves closer examination.

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Abbreviations and terminology

A: photosynthetic assimilation rate
ABA: abscisic acid
d.a.g.: days after germination
δ13C: carbon isotope composition
Δ13C: carbon isotope discrimination
E: transpiration rate
gs: stomatal conductance
MT: Micro-Tom
PCR: polymerase chain reaction
ψ: water potential
RWC: relative water content
SLA: specific leaf area
TE: transpiration efficiency
WELL: Water Economy Locus in Lycopersicon
WUE: water-use efficiency

Chapter 1 – Introduction

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1.1. The problem of agricultural water use

Agriculture is the biggest water consumer in the world, accounting for 70% of the freshwater withdrawals. In the 21st century, however, it will face increasing competition from industrial and domestic water users (Shiklomanov, 2000). Further, climate change is expected to alter the rainfall patterns, affecting rainfed agriculture, which accounts for the livelihood of 852 million people in the developing world (Wani et al., 2009). Thus, the challenge is twofold: on the one hand, to increase the total agricultural output using the same or a reduced amount of irrigation water (Wallace, 2000), And on the other, to develop crops with improved tolerance to water scarcity and better yield under unreliable rainfall patterns (Campos et al., 2004; Cattivelli et al., 2008).

Water is the most limiting resource and yet the most abundantly needed by plants to grow and function efficiently (Boyer, 1982; Kramer and Boyer, 1995). Water makes up most of the mass of plant cells. In each cell, cytoplasm accounts for only 5 to 10% of the cell volume, whereas the remainder is a large water-filled vacuole. The water status of a plant depends on the combined effects of the soil, the atmosphere and the plant itself. Water uptake from the soil is affected by the soil's structure and biophysical properties and by the plant's root system. Water loss from the plant is affected by its internal hydraulic conductance, leaf area, stomatal structure and activity and evaporative demand (determined by atmospheric humidity and temperature). It is therefore no surprise that

such slow progress has been made in understanding the physiology and biochemistry of plant responses to drought, let alone manipulate them through genetic engineering.

There are two different and conflicting aspects to water use by plants. One is related to the unavoidable trade-off between carbon fixation and transpirational water loss by the plants. The ratio of carbon fixed to water lost needs to be increased if more efficient crops are to be bred and a more parsimonious use of the Earth's fresh water is to be achieved. Natural variation exists for water use efficiency (WUE) in plants (Farquhar and Richards, 1984), but the complexity of this trait, which is developmentally controlled and influenced by multiple biological parameters, has hampered efforts to produce more water-use efficient crops (Condon et al., 2004). The second aspect is the response of plants to water scarcity ('drought'), be it in the soil, or in the atmosphere (in the form of water vapour). In agricultural terms, 'drought resistance' is defined in terms of yield in relation to a limiting water supply (Passioura, 1996).

An increased understanding of the physiological mechanisms controlling WUE and drought resistance could lead to increases in agricultural output, and the avoidance of massive agricultural losses during episodes of severe drought.

1.1.1. Water-use efficiency (WUE) and drought resistance

Both WUE and drought resistance are used to designate a large number of phenomena which differ in scale, scope and magnitude. A brief description of those is provided below.

1.1.1.1. WUE

WUE is recognised and defined at various spatio-temporal levels. The most frequently found expressions are intrinsic, instantaneous and long-term (or whole-plant) WUE. The 'intrinsic' WUE (WUE_i) is defined as the ratio between net CO_2 assimilation rate (A) and stomatal conductance (g_s) and was introduced to compare photosynthetic properties independent of (or at the same) evaporative demand (Osmond et al., 1980).

$$WUE_i = A/g_s$$

This definition, however, overlooks the driving force of transpiration, which is leaf-to-air vapour pressure difference (D). The air inside the leaf intercellular spaces is usually assumed to be saturated with water vapour, whereas the vapour pressure deficit (VPD) in the air surrounding the leaf is dependent on temperature and relative humidity, and, unlike either A and g_s taken separately, it is linearly related to evapotranspiration. A more relevant parameter is then 'instantaneous' WUE (WUE_t), also

known as transpiration efficiency (*TE*) where *A* is the numerator and transpiration rate (*E*) instead of gs is the denominator:

$WUE_t = A/E$

Both *A* and *E*, according to Fick's law, are the product of the conductivity, represented by g_s, and the gradient driving the flux, the CO_2 gradient in the case of *A* and the water vapour gradient in the case of *E*:

$A = g_s (p_a - p_i)$

$E = g_s (w_i - w_a)$

where $p_a - p_i$ is the gradient between internal and ambient CO_2 partial pressures, and $w_i - w_a$ the gradient between water vapour mole fractions inside the leaf at leaf temperature and in ambient air, at air temperature. Changes in WUE_i and WUE_t can be uncorrelated. If g_s and *A* are kept constant, a decrease in *E* could reflect increasing atmospheric humidity or decreasing air temperature (*i. e.* decreasing evaporative demand), which would cause an increase in WUE_t but have no effect on WUE_i. On the other hand, if g_s responds to changes in evaporative demand to keep *E* constant, WUE_i would change but not WUE_t.

Further, both WUE_i and WUE_t represent short-term measurements of the physiological processes in the leaf, whereas at longer timescales and at the whole plant level, assimilation and transpiration need to be integrated with other parameters affecting the carbon and

water balance of the plant, namely respiration and 'unproductive' water loss from heterotrophic parts of the plant or nighttime transpiration and cuticular water loss (Farquhar et al., 1989). Thus, 'integrated' WUE (WUE_p) is defined as:

$$WUE_p = \frac{A}{E}\frac{(1-\phi_c)}{(1+\phi_w)}$$

where ϕ_c is the fraction of assimilated carbon lost in respiration and ϕ_w is the fraction of 'unproductive' water loss (*i.e.* not associated to a concomitant gain in fixed carbon). In practical terms, the assimilated carbon can be considered as the total plant biomass (although frequently only aboveground parts are considered) or the economic product (*e.g.* grains), the alternative favoured by breeders and agronomists (Condon et al., 2002; Rebetzke et al., 2002b).

It should be noted that the 'integration' in WUE_p has two components, a spatial and a temporal one. First, dry matter accumulation and water loss take place over a longer time span (days, weeks) than the instantaneous measurements (usually minutes). And second, the measurements are performed in the whole plant, thus including tissues (roots, stems) and processes (cuticular water loss, nighttime transpiration, respiration) which are not taken into account at the instantaneous level. From this it can be inferred that 'scaling up' from intrinsic or instantaneous to integrated WUE is not straightforward. There are instances where increases in WUE_t under controlled conditions

are eliminated in the field (Bolger and Turner, 1998; Lambers et al., 1998). Multiple factors are involved in the transition from the leaf to the plant or canopy level, but the two mains ones are: 1- the boundary layer resistance. If it is high, as in a dense canopy, stomatal opening could exert a lower control over the transpiration rate. Stomatal conductance (g_s) at the leaf level is usually measured under conditions of air turbulence which reduce the boundary layer of unstirred air around the leaves; and 2- a gain in WUE_t brought about by increased stomatal closure would be lower at canopy level than expected from plant level measurements because of higher leaf temperature, which would increase water loss and thus, at least partially, cancel out the benefit of decreased g_s (Condon et al., 2002). Increased leaf temperature can also carry a penalty on CO_2 assimilation through effects on the biochemical machinery of photosynthesis (Schrader et al., 2004). On the other hand, increased leaf temperature can have a positive effect on photosynthesis if the temperature increase moves the leaf closer to optimum temperature, for instance in cooler, temperate climates (Magliulo et al., 2003).

1.1.1.2. Drought resistance

Drought resistance is a more nebulous term than WUE in that it accepts many definitions depending on the timescale (minutes to months), the source of drought (water scarcity in the soil or a large humidity deficit in the air which cannot be met by the supply of water from the soil) and the phenological stage of the plant and the level of

organisation considered, *i.e.* cells, tissues, whole plants (Blum, 2005; Passioura, 1996). The classification of Levitt into drought escape, and dehydration avoidance and tolerance (Levitt, 1972) is still considered the canon and the present discussion is based on his concepts. It should be pointed out, however, that these strategies are not mutually exclusive and plants usually exhibit a combination of them in real-life situations (Chaves et al., 2003). When a genotype yields better than another (in terms of total dry mass or harvestable product) under conditions in which the plant's demand for water is not met by the supply, it is considered more drought resistant. In general terms, three mechanisms of drought resistance are recognised: drought escape, dehydration tolerance and dehydration avoidance, the latter two being classes of drought resistance. In the first, plants rely on ontogenetic alterations to attain the reproductive stage before the onset of a severe stress (Mulroy and Runder, 1977). The hallmarks of this strategy are rapid development and high developmental plasticity, which explains why annual plants tend to favour it. Although their determinate growth habit tends to limit the cereals' developmental plasticity (Fischer and Turner, 1978), selection for rapid development has been the most successful approach in breeding for drought resistance in wheat and barley (Ribaut, 2006). In the mechanism of dehydration tolerance, the plant adjusts its physiological functions to greatly reduced relative water content (RWC) in the tissues and usually enters a quiescent or dormant state until water is again available (Ingram and Bartels, 1996). Little potential is seen in this strategy for breeding drought-resistance into crop species (Vinocur and Altman, 2005). Dehydration avoidance, on the other hand, seems to have been the strategy favoured by both natural

and human selection (Chaves et al., 2002). Avoidance of dehydration encompasses different mechanisms to maintain a high water potential (ψ) in the tissues during a period of increasing soil water deficit or high evaporative demand from the atmosphere, or both (Jones et al., 1981). The result is that the plant avoids being dehydrated, so its physiological functions are left relatively unaffected by the stress. The three possible avenues (not mutually exclusive) to achieve this are: 1- maintenance of water uptake by means of changes in rooting patterns and density (Jackson et al., 2000); 2- reduction of water loss through changes in leaf conductance, absorbed radiation or evaporative surface area (Ehleringer and Cooper, 1992); and 3- osmotic adjustment, which helps maintain a higher RWC at a lower leaf water potential (Jones and Turner, 1978).

It was stated above that WUE and drought resistance are not synonymous concepts, and can in fact sometimes be antagonistic. The reason for this is that the former refers to a measure of productivity, or the optimisation of a ratio of product/resource, whereas the latter concerns coping with a resource limitation and, ultimately, a challenge to productivity and survival (Blum, 2009). Further, since WUE is a ratio, reduced plant growth (which is a ubiquitous consequence of drought) can bring about increases in WUE which are of little practical use. It has been shown that variation in WUE is often associated to variation in the denominator (water use) rather than the numerator (biomass) (Blum, 2005). Plants with extensive root growth contributing to sustained growth and maintenance of high transpiration rates usually show reduced WUE

(Kobata et al., 1996; Pinheiro et al., 2005). In conclusion, increased WUE does not equate with drought resistance and increased drought resistance does not necessarily incur a penalty in yield potential or maximum productivity under non-limiting conditions.

1.2. Biological aspects of the tomato

Tomato (*Solanum lycopersicum*, L.) belongs to the Solanaceae family, which includes other horticultural species, such as potato, pepper and eggplant, as well as medicinal herbs, spices, toxic and ornamental species (tobacco, petunia, various nightshades). As a biological model, tomato shows traits that are not found in other plant systems, like Arabidopsis, such as the development of climacteric fleshy fruit, multicellular glandular trichomes, a profuse secondary metabolism (lycopene and other carotenoids, flavonoids, polyphenols, volatile compounds and other allelochemicals), compound leaf development, photoperiod-independent sympodial flowering, establishment of symbiotic mycorrhizal associations, and agronomically relevant plant-insect and pathogen interactions. Around 30% of the tomato genes have no significant homology to Arabidopsis genes (Van der Hoeven et al., 2002).

The tomato genome spans 950 Mb distributed in 12 chromosomes and is currently being sequenced by the International Tomato Sequencing Project (http://solgenomics.net/genomes/Solanum_lycopersicum/index.pl). A first draft of the genome shotgun sequence is available, with a

complete sequence of the euchromatin (gene-rich regions of the genome). A draft genome sequence also exists of *S. pimpinellifolium*, the proposed wild progenitor of cultivated tomato (http://solgenomics.net/organism /Solanum_pimpinellifolium/genome). Most Solanaceae species have sets of 12 syntenic chromosomes, which reinforces the practicality of using tomato as a model species for other members of this group (such as potato, *Solanum tuberosum*, a tetraploid species).

The Solanaceae genomes are very polymorphic, showing a wide diversity of phenotypes within species (*e.g.*, in size, growth habit, color, shape, other organoleptic properties). This natural genetic diversity can be used as a source of mutants for the discovery of novel traits of economical importance, like resistance to biotic and abiotic stress factors, as well as improved yield and fruit characteristics.

1.2.1. Natural genetic variation in tomato

Genetic variation within a species is an important source of information in any subfield of genetics. Genetic diversity is understood as the evolutionary result of small genomic changes leading to adaptation to diverse natural environments, or in the case of domestication, due to human selection. There are probably more than 10,000 tomato varieties in the world today. Naturally-occurring genetic variation is generally perceived as a better choice of gene selection in breeding programs than artificially generated genetic variation because a certain selective evolutionary pressure has already acted upon the fitness of the organism

(Alonso-Blanco et al., 2005). Dissecting the genetic variation of a species produces a large amount of information with functional, ecological and evolutionary significance for developmental and physiological studies (Alonso-Blanco et al., 2009; Koornneef et al., 2004) and implications for applied breeding programs when studied in crops of economic importance, including tomato (Gur and Zamir, 2004).

The tomato, *Solanum lycopersicum*, is closely related to another 12 species (some of them illustrated in Fig 1) which were all previously part of a separate genus, *Lycopersicon* (Taylor, 1986). Although they share certain traits such as laterally dehiscent anthers and pinnate leaves, the analysis of molecular traits resulted in their placement back in the original Linnean classification within the *Solanum* genus (Peralta and Spooner, 2001). All members of this group are diploids with 12 chromosomes (2n=24) and share a large degree of synteny with one another. Their distribution ranges from southern Ecuador, including the Galápagos Islands, through Perú to northern Chile. This region comprises very different environments, with drylands, areas of high altitudes with low temperatures at night and areas affected by salinity at the sea shore (Taylor, 1986). Each species is adapted to a particular habitat and thus draws interest from breeders with the aim of broadening the genetic base of tomato (Warnock, 1991). *Solanum cheesmaniae*, for instance, is endemic to the Galápagos Islands and is sometimes found as close as 5 m above the hide tide line (Rick, 1973). There, it is subject to salt spray and salt accumulation in the soil, so it is a potential source of genes for salt tolerance (Rush and Epstein, 1976; Tal and Shannon, 1983). *Solanum*

habrochaites is found in a strip of central Perú at altitudes from 500 to 3500 m above sea level. Chilling-resistant ecotypes of this species have been found, as night temperatures at high elevations can drop as low as 5°C (Patterson, 1988; Patterson and Payne, 1983). *S. habrochaites* is also the most notable source of arthropod resistance, although few genes or QTLs have been identified controlling this trait and little progress has been made in breeding these into cultivated tomatoes.

Figure 1. Natural variation in tomato. From left to right, representative leaf and inflorescence of: *S. peruvianum* (LA0153), *S. neorickii* (LA0247), *S. pimpinellifolium* (LA0373), *S. esculentum* var. *cerasiforme* (LA0292), *S. chilense* (LA1930), *S. chmielewskii* (LA1028), *S. pennellii* (LA706), *S. esculentum* cv. M82 (LA3475).

The cultivated tomato is mesophytic, and thus, not significantly resistant to drought. The main sources of genetic variation for drought resistance are the green-fruited wild relatives *Solanum chilense* and *Solanum pennellii* (Rick, 1973). Whereas the former is adapted to one of the most extreme environments on the planet, the Atacama desert (Maldonado et al., 2003), the latter dwells in a narrow strip of 500-1500 m elevation in Central Perú, where the soil is usually dry but the weather is mild (Rick, 1973). The plants of *S. chilense* are gametophytic self-

incompatible, so they are exclusively outbreeders (Rick and Lamm, 1955). There are also several barriers to crosses with *S. lycopersicum* (Martin, 1961). Few seeds are viable and only crossing male *S. chilense* plants with female cultivated tomatoes yields enough viable seeds to facilitate embryo rescue (Chen and Imanishi, 1991). The bipinnate, fern-like leaves of *S. chilense* lose water as rapidly as the cultivated tomato leaves when detached, and have a similarly low ability to withstand desiccation in the entire plant (Rick, 1973). Instead, the drought resistance of this wild species involves the production of extremely long roots which grow deep into the rocky soil of its natural habitat and reach the water tables (Rick, 1973). Leaf area and plant growth rates are reduced under drought, with a concomitant increase in root development (Chen and Tabaeizadeh, 1992). The large investment of *S. chilense* in root biomass is an interesting avenue for research, as significant gains in crop productivity have been obtained in semi-arid regions by breeding for increased root depth (Fischer and Turner, 1978). Several drought-responsive genes have also been cloned from this species (Chen et al., 1993; Chen et al., 1994; Frankel et al., 2003; Yu et al., 1998). In spite of this, the wild species showing the greatest promise for breeding drought resistance into tomato is *S. pennellii* (Rick, 1973; Rudich and Luchinsky, 1986).

1.2.2. *Solanum pennellii* as a source of drought resistance

Solanum pennellii grows in the exceedingly dry western slopes of the Andes, most of its area of distribution lies in rain shadows(Nakazato et

al., 2008; Nakazato et al., 2010; Warnock, 1991; Wong et al., 1979)Throughout its habitat, however, *S. pennellii* experiences ocassional periods of fog (Rick, 1973). Its leaves are small, thick and round, and of a light green colour and sticky texture (Holtan and Hake, 2003). They also have the pecularity of a roughly equal proportion of stomata on the upper and lower surface of the leaf, as opposed to tomato, where most (usually >70%) stomata are found on the bottom, or abaxial, surface (Gay and Hurd, 1975; Kebede et al., 1994). *S. pennellii* has thin, branched roots which grow superficially and amount to less than 5% of the proportional weight in *S. esculentum* (Yu, 1972). It has been reported that crossing *S. pennellii* with *S. lycopersicum* yields a very large root system in the F_1 hybrids, which grows to a greater depth and explores a greater volume than the cultivated parent (Rudich and Luchinsky, 1986).

Ever since Charles Rick showed that *S. pennellii* can be crossed with the tomato, producing a fertile interspecific hybrid (Rick, 1960), plant breeders have been attracted to this species as a potential source of drought resistance and other useful traits. Its leaves are profusely covered with glandular hairs which secrete sticky exudates conferring resistance to insects such as the potato aphid (Gentile and Stoner, 1968) and red spider mite (Gentile et al., 1969). The hallmark trait of the species, is, however, its remarkable ability to withstand water deprivation in the soil (Fig 2). In his unpublished doctoral thesis, Albert Yu (1972) explored some aspects of the water relations in *S. pennellii*. He showed that the water content in fresh *S. pennellii* tissue is considerably higher than in a tomato cultivar (VF-36). He also proved that the difference in water loss from detached

leaves was negatively correlated to stomatal density and thus, that regulation of stomatal opening could be the key factor determining water use. Heterotic performance was observed for the F_1 interspecific hybrids of *S. pennellii* and tomato for water-use efficiency (WUE) and for the percentage water loss from detached leaves (Yu, 1972). The latter was decreased and the former increased in the hybrid with respect to either parent. One further study, unfortunately also unpublished, compared water relations of the tomato, *S. pennellii* and their mutual F_1, confirming several of the observations made by Yu (Cohen, 1982).

It was subsequently shown by other researchers that *S. pennellii* also has a higher WUE, defined as the amount of carbon fixed by the plant per unit of water transpired. This trait was shown to be under genetic control and F_1 plants of crosses between *S. pennellii* and cultivated tomato showed intermediate WUE values between the parents (Martin and Thorstenson, 1988). Three QTL controlling WUE were later identified (Martin et al., 1989) and more recently, a QTL for WUE was detected in the *Solanum pennellii* chromosome fragment of IL5-4, an introgression line with *S. lycopersicum* cv. M82 background (Xu et al., 2008).

The drought resistance of *S. pennellii* has also been studied at the genetic and biochemical level. Kahn et al. (1993) showed that in detached leaves that were wilted to 88% of their fully-turgid weight, *S. pennellii* maintained a higher leaf water potential and accumulated less ABA than *S. lycopersicum* or hybrids of the two species. Drought-responsive genes (encoding an H1 histone and lipid transfer proteins) have been cloned

from *S. pennellii* (Treviño and Connell, 1998; Wei and O'Connell, 1996). The drought-induced H1 histone has been suggested to function in regulation of changes in gene expression in response to drought stress, whereas the lipid transfer proteins are believed to function in the deposition of thicker wax layers (O'Connell et al., 2007).

Figure 2. Resistance to wilting in *S. pennellii*. Plants of *S. pennellii* (left) and tomato cv. M82 (right) were grown in the same pot. At the stage of seven leaves, water was withheld and the photograph taken five days later Height of pot: 25 cm

1.2.3. The Micro-Tom cultivar as a biological model system

Micro-Tom (MT; Fig 3) is a dwarf cultivar of tomato, allowing a planting density of up to 1,300 plants/m^2. It usually grows 15 cm tall, compared to 1 m and more in commercial varieties. Scott and Harbaugh (1991) originally described MT as an ornamental variety, but it was later proposed as a convenient genotype for functional genetics studies (Meissner et al., 2000). Since then many studies have used MT to address a wide range of problems in plant biology (Campos et al., 2009; Isaacson et al.,

2002; Lima et al., 2004; Meissner et al., 1997; Serrani et al., 2007; Tieman et al., 2001). An extensive discussion on the use of MT as a model plant system was published recently (Campos et al., 2010).

Figure 3. *Arabidopsis thaliana* and Micro-Tom tomato (*Solanum lycopersicum*). Both plants grown in 350 mL pots and photographed at flowering. Height of pot: 10 cm.

The MT cultivar harbours several mutations absent in commercial cultivars. The best known mutant alleles are: *dwarf* (*d*), a brassinosteroid-related mutation responsible for the small plant size,located on chromosome 2, (Bishop et al., 1999), and *self-pruning* (*sp*), responsible for its determinate growth habit, on chromosome 6 (Marti et al., 2006; Pnueli et al., 1998). The *miniature* (*mnt*) allele was also suggested to contribute to the MT small plant size (Meissner et al., 1997), although this has not been yet proven. Additional reported alleles present in MT are *uniform ripening* (*u*; chromosome 10), *Stemphylium resistance* (*Sm*; chromosome 11) and *Immunity to Fusarium wilt* (*I*; on chromosome 7) (Scott and Harbaugh, 1991).

1.4. Aim of this work

The question addressed in this work is whether the well-known drought resistance of *S. pennellii* has a monogenic component which could be introgressed into cultivated tomato and inherited stably therein without significant penalty to either yield or plant growth. A direct genetics approach is used of crossing and screening for delayed wilting under water deprivation. Unlike previous work where the physiology of drought resistance in *S. pennellii* was studied in F_1 interspecific hybrids (Cohen, 1982; Yu, 1972) the aim here is to go one step beyond and produce true-breeding lines with increased resistance to drought. In the eventuality of finding such a line, the next objective is to test its WUE under well-watered and drought conditions and perform a physiological characterisation of the novel line to determine the mechanistic basis for its increased resistance to drought, which in *S. pennellii* is known to be a combination of stomatal density, distribution and dynamics (Kebede et al., 1994). Finally, one further aim is to narrow down the introgression from *S. pennellii* to the smallest possible segment and look for candidate gene(s) in the newly-created line.

Chapter 2 – Introgression of drought resistance from *Solanum pennellii* into *S. lycopersicum* cv. Micro-Tom

2.1. Introduction

The wild relative of tomato *S. pennellii* (LA716) was crossed to *S. lycopersicum* cv. Micro-Tom (MT), using the latter as the female parent. The F_1 hybrids were all tall, although shorter than the wild species parent, and showed an indeterminate growth habit. The F_1 plants were backcrossed (BC_1) with the recurrent parent MT, which was used as the female in this and all subsequent crosses (Fig 1).

Approximately 600 BC_1 plants were grown in germination trays, alongside 30 wild-type MT plants grown in individual 100-ml pots. Two weeks after germination a visual screen was performed on the BC_1 seedling population to select miniature plants of similar size to MT. Forty plants were selected and transplanted to pots, the rest were discarded. At the onset of flowering, between 30 and 35 days after germination, watering was withheld in both the selected BC_1 and MT. plants were inspected daily for wiltiness, and when all 30 MT individuals had lost turgidity and become droopy, six of the original 40 BC_1 plants still showed only minor signs of wilting. The most turgid of these six plants was re-watered and selfed to produce seeds, but it produced only seedless fruits and was thus used as a male parent in a second backcross with MT (BC_2).

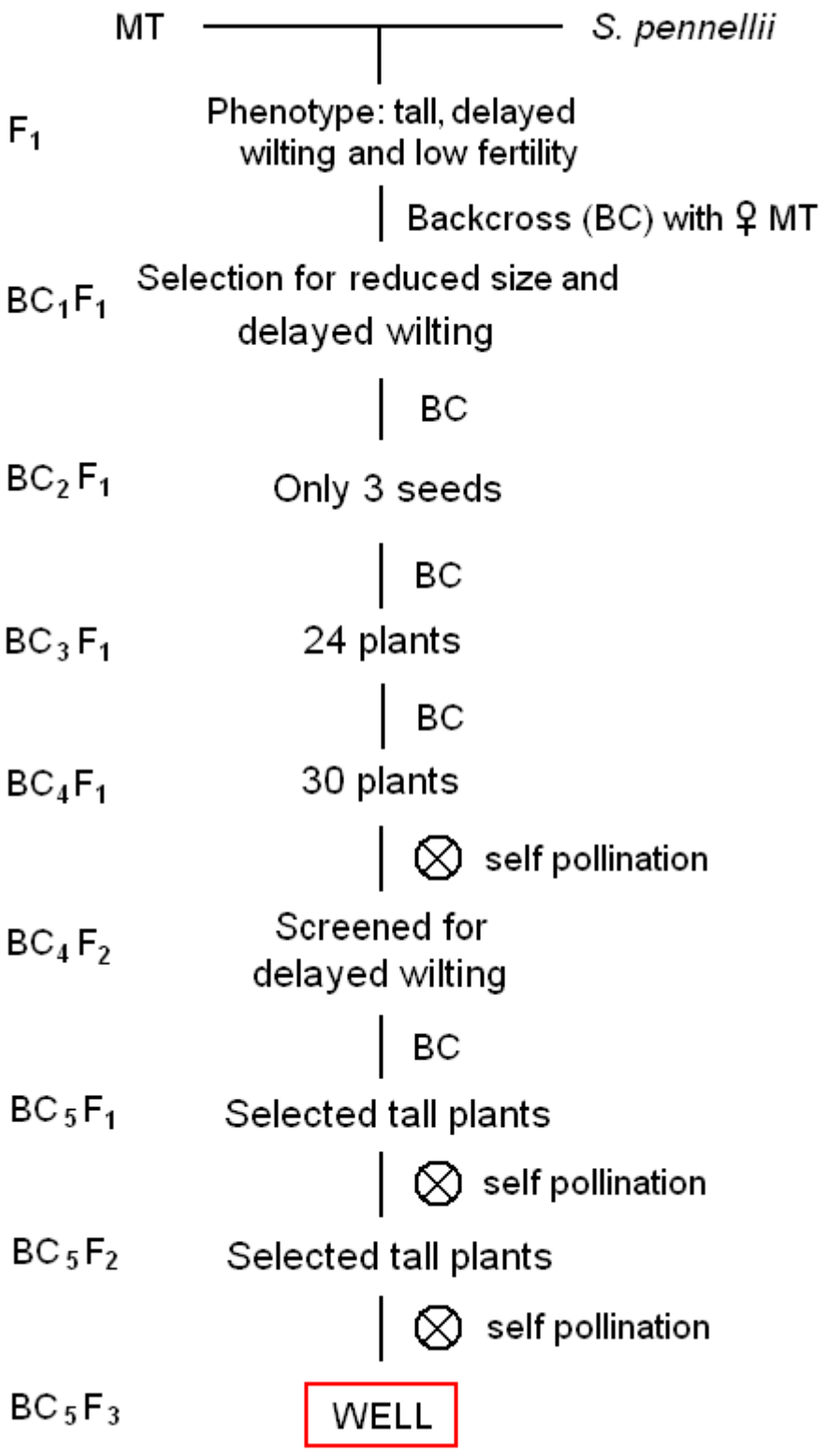

Figure 1. Diagram illustrating the breeding procedure used to create the new introgression line (WELL) by crossing *Lycopersicum esculentum* cv. Micro-Tom (MT, LA3911) with *Solanum lycopersicum* (LA716). Refer to text for details.

A BC_2 plant was the male parent in the next backcross (BC_3), a bottleneck which posed the risk of losing the non-wilty genotype. A pollen mix from 24 BC_3 plants was used to fertilize MT again (BC_4) and the 30 resulting plants were cultivated and selfed (BC_4F_2). Twenty-four plants from this generation were grown, each paired with one MT plant in a 350 ml pot (Fig. 2). The rationale for this was to expose the roots of both plants to similar water supplies. When both plants in the pot had flowered, watering was stopped. Wilting was assessed visually and by touching the leaves. All five of the BC_4F_2 plants with delayed wilting were taller than their wilty MT counterparts. A pollen mix from those five plants was used to fertilise MT again (BC_5). From these plants, the tall offspring (approximately 10 out of 24) were selfed to produce BC_5F_2 seeds.

Figure 2. Screening of BC_4F_2 plants using the single pot screening method (see text). Left: BC_4F_2 plant. Right: MT. Photo taken after 5 days of water withdrawal.

BC_5F_3 families were screened for the absence of short segregants to identify BC_5F_2 homozygous lines. Plants in such lines are true-breeding and could at this point be considered near-isogenic to the recurrent parent MT (Stam and Zeven, 1981). This line was named WELL, an acronym for Water Economy Line in Lycopersicon.

2.2. Methods

Seeds of *S. pennellii* LA716 were kindly donated by Dr. Roger Chetelat (Tomato Genetics Resource Center, Davis, USA) and seeds of *S. lycopersicum* cv. Micro-Tom (MT) by Dr. Avraham Levy (Weizmann Institute of Science, Rehovot, Israel). All seeds were surface-sterilized by treatment with a 5% v/v solution of household bleach (White King, Australia) for 5 minutes and then rinsed with distilled water. Seeds were sown in 40x20x5 cm trays filled with seed raising mix. Upon the appearance of the first pair of true leaves, seedlings were transplanted to pots filled with either pasteurized coarse sand or a 50/50 v/v mixture of seed raising mix and vermiculite, as indicated below. Plants grown in glasshouse (350 ml pots filled with 1:1 mixture of seed raising mix and vermiculite supplemented with 1 g L^{-1} 10:10:10 NPK and 4 g L^{-1} lime; sunlight 250-350 μmol photons m^{-2} sec^{-1} PAR; 11.5h photoperiod; 30/26°C temperature day/night and 60-75% ambient relative humidity).

2.3. Results

2.3.1. The WELL line exhibits several distinctive phenotypes

As mentioned above and illustrated in Figure 2, WELL plants, besides showing delayed wilting, were significantly taller than MT. In order to characterise more in detail the phenotype of WELL, an WELL plants were grown alongside MT will be set up. The plants were cultivated

under glasshouse conditions and harvested at the beginning of fruit set (around 90 days after germination). Phenotypic observations were performed to compare both lines.

2.3.2. WELL plants are taller than MT and semi-determinate

WELL plant height, measured 62 days after seed germination as the distance from the soil to the top of the highest plant node, was approximately twice that of MT (Fig 2). This increase was caused by more elongated internodes (Table 1).

Table 1. Comparison of internode length and plant height (mm, measured from soil to the top of the highest node) between MT and WELL plants at maturity, 62 days after seed germination. Mean ± s.e.m.(n=5).*p*-values calculated with a *t*-test. * and ** indicate significant differences at $p<0.05$ and $p<0.001$ respectively.
p-value calculated with a *t* test: * and ** indicate significant differences at $p<0.05$ and $p<0.01$ respectively.

Internode between leaves	MT	WELL	*p*
1-2	4.7 ± 1.4	14.7 ± 2.4	0.0121 *
2-3	10.5 ± 1.2	23.2 ± 2.1	0.0021 **
3-4	14.2 ± 2.6	24.5 ± 1.80	0.0194 *
4-5	14.5 ± 1.5	29.7 ± 0.7	0.0001 **
5-6	18.5 ± 1.0	32.5 ± 4.2	0.0245 *
Plant height	110.0 ± 3.8	245.0 ± 5.3	0.0001 **

There was also significantly more vegetative growth after flowering in WELL, with the production of 13 leaves on average in WELL plants against only 9 in MT (Fig 3). The termination of apical growth via the formation of two consecutive inflorescences characteristic of the determinate tomato was delayed in WELL (Fig 3).

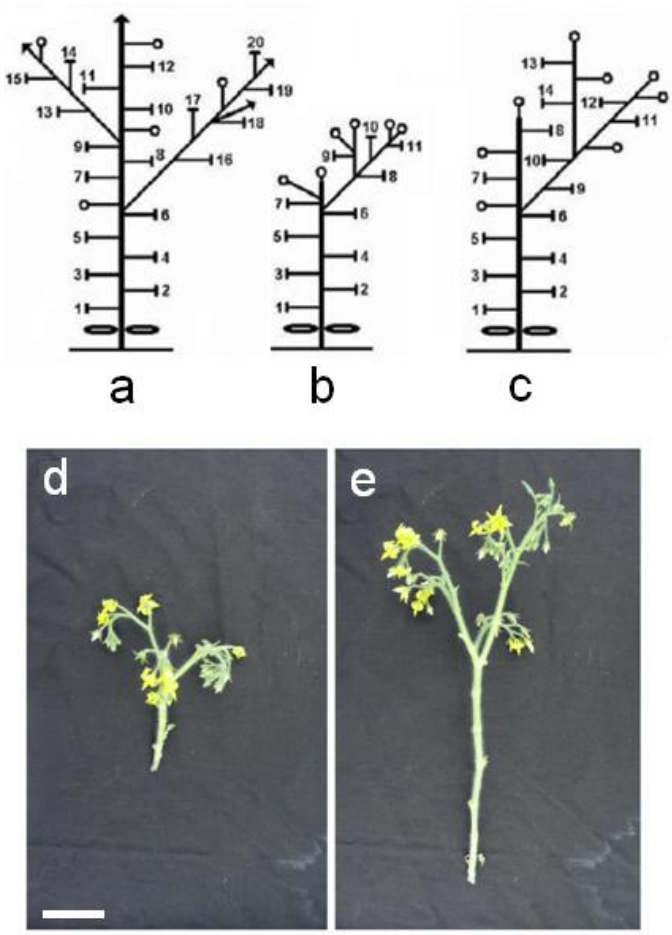

Figure 3. Top: Schematic representation of indeterminate (a), determinate (b) and semi-determinate (c) growth habit in tomato cv. Micro-Tom. "T" bars represent leaves with 5 leaflets (except for leaves 1 and 2, which have 3 leaflets), small circles represent inflorescences with 7 flowers and arrows represent growing shoots. The schemes were based on the observation of 10 plants from the following genotypes: MT-SP BC_6F_n (indeterminate, see next chapter for details about this genotype), MT and WELL BC_5F_n. The MT-SP line harbours the *SP* allele as opposed to the *sp* allele present in MT and WELL. Bottom: Stems of representative (d) MT and (e) WELL plants with all leaves removed for ease of visualization. Bar = 5 cm.

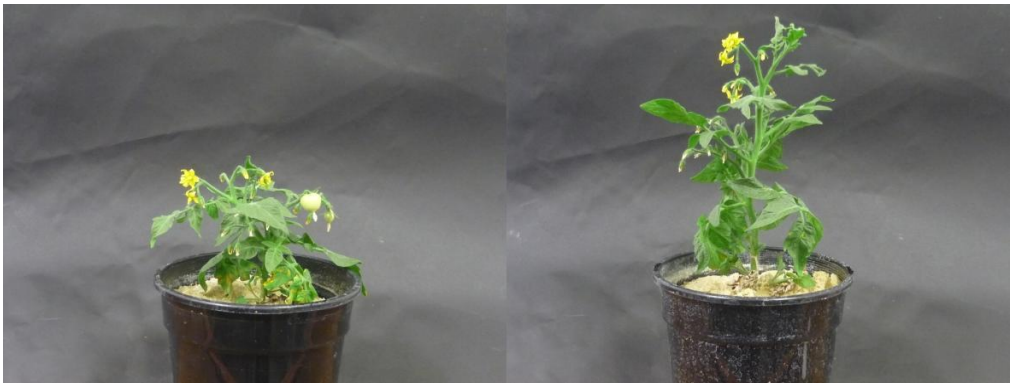

Figure 4. Comparison of representative MT (left) and WELL (right) plants 45 days after germination.

Consistent with a semi-determinate growth habit, WELL plants produced more inflorescences and fruits than MT (Fig 5, Table 3D).

Figure 5. Representative MT (left) and WELL (right) plants at maturity (90 days after germination). Notice the higher number of fruits in WELL.

2.3.3. WELL leaves are more erect and wrinkled

Another distinctive feature of WELL was its more erect leaves. This was already evident for the first pair of true leaves at the seedling stage (Fig 6). In full-grown plants, leaf insertion angle was approximately 45° for MT (n=6), compared to 30° for WELL (n=6). In addition, WELL leaves appeared slightly more wrinkled (Fig 7)..

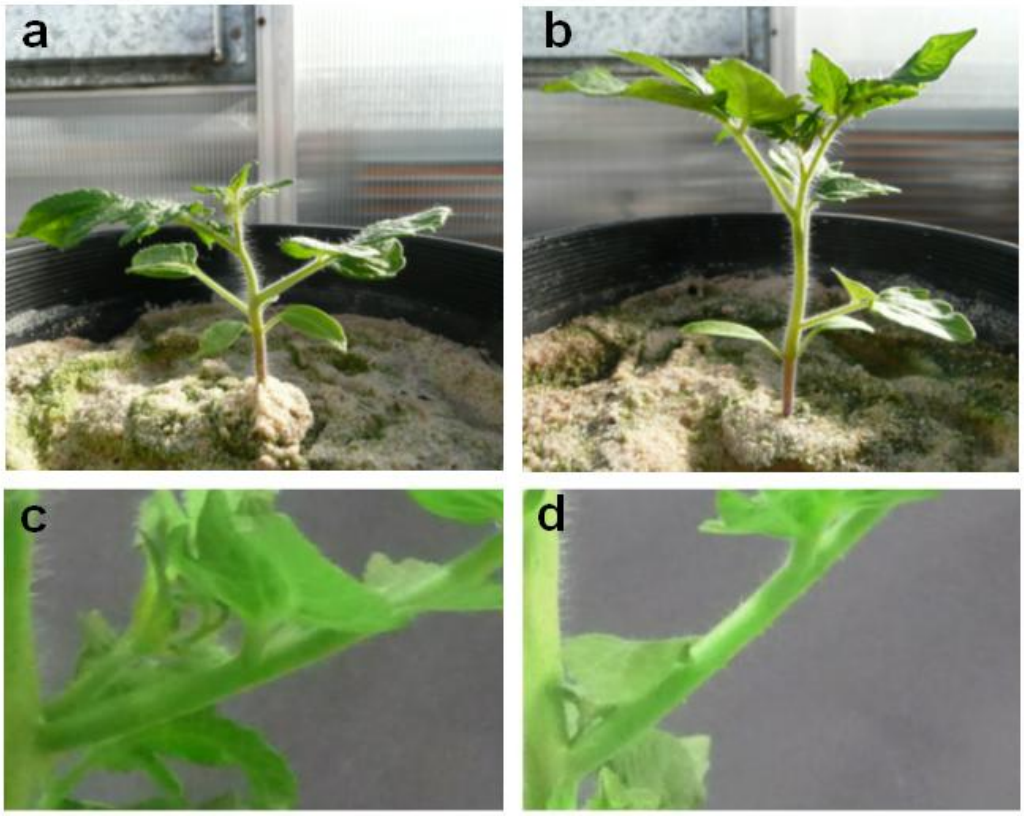

Figure 6. Top: 25-day old plants of (a) MT; (b) WELL. Bottom: close-up showing representative leaf insertion angle in 50-day old plants of (c) MT and (d) WELL.

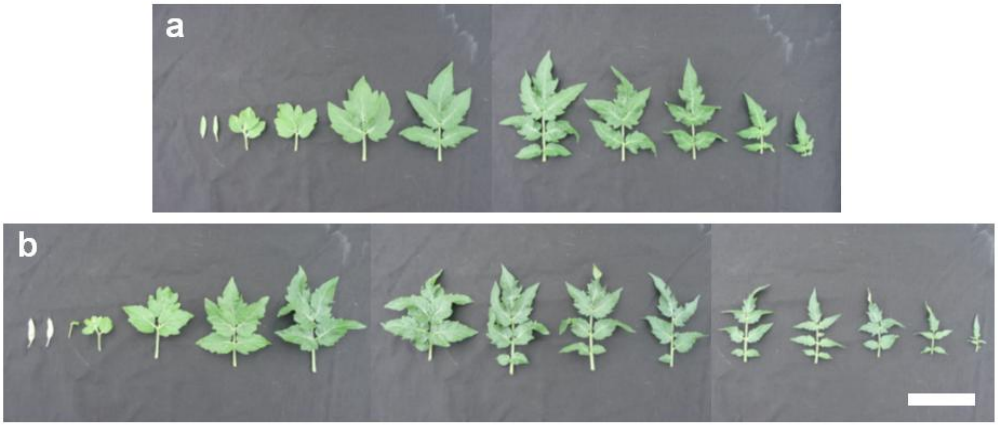

Figure 7. Detached primary leaves (growing on the main stem) from representative full-grown (a) MT and (b) WELL plants. Leaves were detached from 50-day-old plants grown in well-watered conditions. Bar = 5 cm.

2.3.4. WELL has pink fruits

The ripe fruit of WELL did not attain the characteristic intense red colour of its recurrent parental MT, but rather lingered on a pinkish hue. Upon peeling and visual examination, the WELL fruit epidermis appeared transparent, as opposed to the normally yellow colour when observed through transmitted light (Fig. 8). This fruit phenotype was originally described in 1925 as a monogenic recessive mutation leading to the formation of a colourless fruit peel, and was named "*y*" as the recessive colourless allele of the dominant yellow "y^+" allele (Lindstrom, 1925). In 1956, the *y* mutation was mapped by linkage analysis to the short arm of chromosome *1* (Rick and Butler, 1956). The colourless fruit epidermis is found in numerous cultivated varieties and most wild tomato species, including *S. pennellii*. This provided a strong indication that the *S. pennellii* introgression in WELL could be located on chromosome *1*.

Figure 8. Segment of epidermes peeled from ripe fruit of MT (left) and WELL (right).

2.4. Discussion

The model tomato cultivar MT was crossed to the wild relative *S. pennellii*, as a potential donor of genetic determinants enhancing drought resistance and the efficiency of water use for growth. After several rounds of backcrosses to MT and screening based on plant height and propensity not to wilt under limited water supply, a promising introgression line was recovered, characterised by a significant delay in leaf wilting upon withdrawal of watering, as well as a semi-determinate growth habit, and pink fruits. This line was named WELL, an acronym for Water Economy Line in Lycopersicon.

The increased size of WELL plants combined with their delayed wilting suggested the possibility that WELL, as its wild parent *S. pennellii*, may have enhanced long-term water-use efficiency (WUE), *i.e.* increased amount of dry mass per unit of water transpired (Martin and Thorstenson, 1988; Yu, 1972).

2.4.1. Factors potentially affecting the delayed wilting of WELL

The turgidity of a plant is maintained by the capacity of tissues to hold a sufficient amount of water. Water is in a dynamic state of continual motion, from the soil, through the plant and to the atmosphere in the form of vapour. Thus, unless the movement of water is closely regulated, so that as water leaves a cell it is replaced by an equivalent quantity, fluctuations will result in variations in the state of turgidity of the plant.

Inability of the cell to replenish water loss results in wilting. It follows from this that the factors influencing turgidity and wilting are those controlling a plant's water uptake and water loss. At a given soil water potential (ψ_{soil}), leaf water potential (ψ_{leaf}) is given by the following equation:

$$\psi_{leaf} = \psi_{soil} - \rho g h - E/K_{plant} \qquad Eqn\ 1$$

which is Ohm's Law analogy for the soil-plant-atmosphere hydraulic continuum (van den Honert, 1948). E, ψ_{soil}, ρ, g, h, and K_{plant} represent, respectively, transpiration rate, soil water potential, density of water, acceleration due to gravity, plant height and whole-plant hydraulic conductance. Leaf turgor is given by:

$$P = \psi_{leaf} - \pi \qquad Eqn\ 2$$

where P and π denote turgor and osmotic pressure, respectively.

At a given ψ_{soil}, a difference between MT and WELL in their ability to maintain turgor could arise from differences in any or all of at least three parameters: transpiration rate, hydraulic conductance and osmotic

potential. Each of these is affected by a combination of factors which are described in more detail below.

The transpiration rate of a leaf is a measure of the evaporative flux density (E) of water vapour from the cell walls within the leaf to the outside air. This flux is governed by Fick's first law of diffusion of gases in air (Fick, 1855):

$$E = g_L (w_i - w_o) \qquad \textit{Eqn 3}$$

where g_L is the leaf conductance and (w_i-w_o) is the gradient driving the flux, determined by the difference between the mole fraction of water vapour at the evaporative surface inside the leaf (w_i) and the mole fraction of water vapour in the ambient air surrounding the leaf (w_o). As can be deduced from Equation 3, the only parameter under control of the plant is g_L, whose reciprocal is leaf resistance (r_L). Water vapour must cross a series of leaf components encountering resistance at each step, so the pathway is better described as series of resistances (van den Honert, 1948). The four main sources of resistance to the flow of water vapour during transpiration are: a) intercellular air spaces, b) the leaf cuticle, c) the boundary layer adjacent to the leaf, and d) stomatal pores. The irregular shape of the intercellular air spaces makes it difficult to estimate accurately the resistance contributed to water vapour flow, but this value is usually considered to be very low. *S. pennellii* leaves appear to have more developed air spaces within the mesophyll than *S. lycopersicum* (Kebede et al., 1994). The waxy cuticle covering the leaf surface is a very

effective barrier to water movement, so that less than 5-10% of water loss from the leaves occurs via cuticular transpiration (Holmgren et al., 1965). To reach the atmosphere water vapour must also diffuse through the layer of unstirred air adjacent to the leaf surface. The resistance provided by this boundary layer is proportional to its thickness, which is determined mainly by wind speed (Nobel, 2009). Various anatomical and morphological aspects of the leaf can alter the effect of wind on the thickness of the boundary layer. The shape and density of leaf trichomes, the sunken or exposed position of the stomata and the size and shape of the leaves, all influence the way the wind sweeps across the leaf surface (Gutschick, 1999; Nobel, 2009). The trichomes of tomato and its wild relatives were first examined by Luckwill in 1943 and classified as glandular and non-glandular in seven types, I-VII (Luckwill, 1943). *S. lycopersicum* has all trichome types except II and IV, whereas *S. pennellii* lacks trichomes type II and V, type IV being the most abundant in this species (Lemke and Mutschler, 1984). Trichome density and distribution in tomato and its wild relatives has attracted interest due to its breeding potential against arthropod pests (Simmons and Gurr, 2005), but little is known about their role in the gas exchange properties of the leaf (Benz and Martin, 2006). The increased wrinkling of WELL leaves could, at least in part, contribute to a higher boundary layer resistance and thus lower transpirational water loss.

The main control on transpirational water loss from the leaf is exerted by the stomata. Various factors relay information about the water status of the plant to the stomata, which act as hubs determining the

optimal degree of aperture at any given time (Farquhar and Sharkey, 1982; Hetherington and Woodward, 2003): first, environmental factors, such as CO_2 concentration, light and air humidity, all affect stomatal movement in a way that tends to ensure that conductance keeps pace with the need for CO_2 inside the leaf (Wong et al. 1979); second, a complex of hormones, namely ABA (Jones and Mansfield, 1970; Little and Eidt, 1968), cytokinins (Blackman and Davies, 1985; Tanaka et al., 2006), auxins (Mansfield, 1967; Pemadasa and Jeyaseelan, 1976; Tal et al., 1974) and ethylene (Desikan et al., 2006; Tanaka et al., 2005; Taylor and Gunderson, 1986). ABA is also relevant to plant water relations through regulation of gene expression (Kahn et al., 1993) and hydraulic conductivity (Thompson et al., 2007) of the plant upon drought stress; third, hydraulic linkage, or information conveyed to the stomata about the bulk water status of the plant (Buckley, 2005) and soil water potential (Gollan et al., 1986; Zhang and Davies, 1990), but also as a response to changes in air humidity altering evaporative demand (Farquhar, 1978; Lange et al., 1971).

Leaf conductance to water vapour correlates positively with stomatal density, the number of stomata per unit leaf area (Muchow and Sinclair, 1989; Reich, 1984). The density and distribution of stomata vary between the upper (adaxial) and lower (abaxial) face of leaves. *S. pennellii* is an amphistomatic species, *i.e.* it has similar stomatal densities on the adaxial and abaxial side of the leaf, whereas cultivated tomatoes are hypostomatic, with more than 70% of the stomata found on the abaxial side of the leaf (Gay and Hurd, 1975). The total combined number

of stomata for both sides is also lower in the wild species than in tomato (Kebede et al., 1994). Although the physiological significance of amphistomaty is not yet clear, one long-known trend is the presence of this type of leaf in dry habitats with high irradiance (Wood, 1934). It has been suggested that distributing stomata between two surfaces rather than one may raise maximum leaf conductance to CO_2 while doubling the boundary layer resistance for the flow of water vapour outside the leaf (Mott et al., 1982). Stomatal density appears to be a very plastic trait in tomato, strongly affected by environmental factors. Increasing light intensity five-fold under glasshouse conditions was found to drive adaxial stomatal density up from one stomata per mm^2 to more than 30 and from 80 to 100 per mm^2 on the abaxial side of a tomato leaf (Gay and Hurd, 1975). Water withdrawal for seven days led to lower stomatal densities on the abaxial side of leaves developed during the drought stress period (Sam et al., 2000). Increasing air humidity from a vapor pressure deficit of 1.0 KPa to 0.2 KPa had the opposite effect, leading to a considerable increase in abaxial stomatal density (Bakker, 1991). When grown in similar environmental conditions *S. pennellii* had a 29% lower average stomatal density and lower stomatal apertures than *S. lycopersicum* (Kebede et al., 1994). The wild species also has higher instantaneous water use efficiency (WUE_i), which is the ratio between carbon fixation (*A*) and transpirational water loss (*E*) measured at the leaf (Kebede et al., 1994). A number of genes have been identified in Arabidopsis which affect stomatal development, stomatal density and stomatal dynamics (reviewed in Rowe and Bergmann in 2010 and Kim et al., 2010). Considerably less is known about this in tomato, although with the recent

publication of the first draft of the complete tomato genome sequence (http://www.sgn.cornell. edu) it is expected that this gap will be filled, taking advantage of synteny and sequence homology between tomato and Arabidopsis (Fulton et al., 2002; Ku et al., 2000).

Leaf hydraulic conductance (K_{leaf}) is a measure of how efficiently water is transported through the leaf. K_{leaf} is highly dynamic and can respond rapidly to changes in temperature, irradiance and water supply (Sack and Holbrook, 2006). Thus, it is possible that differences in wiltiness between MT and WELL are a consequence of differences in $K_{leaf.}$ Although the resistance of open stomata to water vapour diffusion out of the leaf is hundreds of times greater than the hydraulic resistance to bulk flow of water through the plant, the maintenance of open stomata depends on having a high leaf water potential (ψ_{leaf}). Leaf water potential, in turn, is related to the hydraulic conductance of the plant (K_{plant}) as shown in Equation 2. It follows from that equation that for a high ψ_{leaf} to be sustained, and hence stomatal opening, K_{plant} has to be sufficiently high. K_{plant} is largely determined by K_{leaf}, as up to 80 to 98% of the hydraulic resistance in whole plants ($R_{plant} = 1/K_{plant}$) is contributed by the leaf (Brodribb et al., 2002; Nardini and Salleo, 2000). Root hydraulic conductivity (K_{root}) however, can also influence shoot water relations considerably, and this has been shown for tomato under salinity stress (Rodriguez et al., 1997). K_{leaf} decreases considerably during water stress, driving stomatal closure and releasing the tension in the transpiration stream (Cochard et al., 2002; Nardini and Salleo, 2000). This mechanism appears to work as a

'safety valve' to prevent xylem embolism and cavitation (Sack and Holbrook, 2006). Intrinsic differences in K_{leaf} between MT and WELL could account for the improved response to drought in the latter.

Yet another parameter which can affect plant turgor is osmotic potential (π). This is a measure of the potential of water to move between regions of different solute concentrations across a semi-permeable membrane such as the plant cell's membrane. By definition, the water potential of pure water is zero. Adding solutes to pure water will lower the water potential, so that through a semi-permeable membrane pure water will tend to move in the direction of the solution and not vice versa. For this reason the value of is π always negative. In the same fashion, as a plant depletes soil water a reduction in cellular π can enhance its ability to take up more of it. Changes in π can be achieved through changes in symplastic water content (V) or in the number of moles of solute in the symplasm (n), because:

$$\pi = (nRT)/V \qquad \textit{Eqn 4}$$

where R is the gas constant, and T is Kelvin temperature. A reduction in π caused by a net accumulation of solutes is called osmotic adjustment (Bernstein, 1961; Bernstein, 1963). As explained above, lower π results in

water being attracted to the cells and tends to maintain *P* above zero (Equation 2) which is a necessary condition for growth to continue (Blum, 1996). This also allows the maintenance of open stomata, and thus higher net rates of photosynthesis at lower values of ψ_{leaf} than otherwise would be possible as ψ_{leaf} falls. One of the consequences of osmotic adjustment is then that plant growth can continue under conditions of soil water scarcity (Green and Cummins, 1974). Osmotic adjustment is under genetic control (Zhang et al., 1999) and has been shown to result from the biosynthesis of organic compounds, such as proline or betaine, upon the onset of water stress (Hsiao et al., 1976). *S. pennellii* was shown to accumulate more proline than cultivated tomato when grown in soil treated with polyethylene glycol6000 to induce water stress (Perez Alfocea et al., 1993; Tal et al., 1979). The problem with exposing plants to a non-diffusible substrate is that it does not represent the situation under natural conditions. To overcome this drawback, Torrecillas et al. (1995) subjected both tomato and *S. pennellii* to seven days of water withdrawal followed by re-irrigation and performed various measurements after plant recovery. They observed a reduction in osmotic potential values indicative of osmotic adjustment through accumulation of reducing sugars and proline only in the cultivated species. The composition of the cellular osmotic solution remained constant in *S. pennellii* throughout the drought treatment and recovery period (Torrecillas et al., 1995).

Screening on the rate of wilting was done following water withdrawal after the onset of flowering, when the semi-determinate

WELL was still producing primary leaves while the determinate MT had terminated apical growth and was producing flowers and lateral branches. A lower total transpiring area in WELL cannot be ruled out, if individual leaves are expanding at a lower rate or expanding to a smaller final size than in MT. The wild relative *S. pennellii* has characteristically small leaves compared to cultivated tomatoes (Holtan and Hake, 2003) and it is possible that at least some of the multiple quantitative trait loci (QTL) known to control this trait have been introgressed in the WELL line.

2.4.2. Wilting, drought resistance and water-use efficiency (WUE)

Drought resistance and WUE are not synonymous (Blum, 2005). Delayed wilting could be a consequence of a more conservative use of water, and hence improved drought resistance. But this does not *per se* imply a higher WUE. For this to be true a plant would need to bring about a decrease in water use with no decrease, or only a minor one, in CO_2 fixation, thereby increasing the ratio of carbon fixation (A) to transpirational water loss (E). As explained in Section 2.3.1, plants usually tend to coordinate A and g_s (Wong et al., 1979), but genetic variation in their ratio has been demonstrated in a range of species, including tomato (Knight et al., 2006; Martin and Thorstenson, 1988; Nienhuis et al., 1994). *S. pennellii* has long been known to have a higher WUE than cultivated tomato (Yu, 1972). The advantage in favour of the wild species is increased when plants are grown under reduced soil moisture (Martin and Thorstenson, 1988). WELL needs to be examined for this trait, as WUE is

critical to plant performance under limited water supply. Scoring on wiltiness was done following water withdrawal, *i.e.* the timing of visual wilting under conditions where plants had to rely on a similar, finite amount of water stored in the pot when last watered. Under these conditions, if the *S. pennellii* introgression brought about higher WUE while not enhancing growth rate, one would expect WELL leaves to wilt later.

Several mechanisms affecting cellular turgor may be involved in the delayed wilting of WELL. Loss of turgor, however, does not always translate into wilting. In many species, leaves do not wilt when water stressed (*e.g.* eucalyptus) or roll rather than wilt (various grasses) due to the mechanical properties of specialised bulliform epidermal cells, or to high silica content, as in rice (Esau, 1977). It cannot therefore be excluded that the *S. pennellii* introgression in the WELL line causes delayed leaf wilting in part through developmental or structural changes in leaves rather than solely through effects on maintenance of cellular turgor.

2.4.3. Water relations and plant architecture

In many plant species the shoot apical meristem (SAM) is indeterminate, thus, it is active during the entire life span of the plant, first producing leaves and, upon the appropriate photoperiodic cues, flowers. This growth behaviour where vegetative and reproductive phases are clearly separated is termed monopodial (Schmitz and Theres, 1999). In contrast, an alternation of vegetative and reproductive phases is observed

in the Solanaceae, a growth behaviour referred to as sympodial. In these species, the SAM terminates after producing a module (called a sympodial unit or sympodium) of three leaves and an inflorescence. Development continues in a relay fashion from lateral meristems, whose vigorous growth displaces the terminal inflorescence, giving the impression of an upright continuity in the stem. Thus, although each individual meristem is determinate (because they terminate after producing one sympodium), the wild-type plant growth habit is 'indeterminate', because the shoot is composed of sympodia which can add up indefinitely (Pnueli et al., 1998). It has been suggested that the more advanced monopodial shoot evolved from the sympodial pattern through a loss of side branches, and that the sympodial shoot, in turn, evolved from an earlier dichotomous branching model via sequential loss of branching (Stewart, 1964).

The indeterminate state of the SAM in monopodial species such as Antirrhinum and Arabidopsis is maintained by the genes *CENTRORADIALIS* (Bradley et al., 1996) and *TERMINAL FLOWER* (Alvarez et al., 1992), respectively. A recessive mutation in the *SELF PRUNING* (*SP*) gene, the orthologue of *CEN* and *TFL* in tomato, produces a progressive reduction in the number of leaves in the sympodial units, so that the shoot eventually terminates in two successive inflorescences without intervening leaves (Macarthur, 1932; Pnueli et al., 1998; Yeager, 1927). The switch to 'determinate' growth habit in *sp* mutants results in compact plants with more homogeneous fruit set, yielding a greater proportion of ripe fruit at harvest. This is due to both the early termination of apical growth and the reduction in internode length in the

mutant (see Table 1). Most modern processing tomato varieties are determinate, as this favours mechanical harvesting (Marti et al., 2006; Stevens and Rick, 1986).

The tomato *SP* gene has been cloned and shown to be a member of a small family, which includes *CEN* and *TFL*, that shares sequence similarity with a group of mammalian phosphatidylethanolamine binding proteins (Grandy et al., 1990; Pnueli et al., 1998). It was subsequently proven that they are functionally different to their mammalian counterparts, and they were included in a new protein family, called *CETS* (Pnueli et al., 1998). The molecular nature of these proteins remains elusive. *CETS* genes do not appear to encode DNA binding proteins, transcription activators, kinases or receptors and they have no effect on cell survival or fate (Pnueli et al., 2001).

Tomato harbours at least five *SP* paralogs (*SP2I*, *SP3D, SP5G*, *SP6A* and *SP9D*), which share homology among themselves and with the six members of the Arabidopsis *TFL* family (Carmel-Goren et al., 2003). Allelic variation for each of these genes exists in the wild species *S. pennellii* (Carmel-Goren et al., 2003). The candidate phenotypic variation associated with these alleles can be studied using the collection of introgression lines (ILs) of *S. pennellii* in *S. lycopersicum* cv. M82 (Eshed and Zamir, 1995). Plants of the wild species *S. pennellii* are also indeterminate, but with two rather than three nodal leaves per sympodium. The introgression containing the *S. pennellii SP9D* allele was associated with 'semi-determinate' growth, where the shoot of *sp*/*sp*

SP9D^{pen}/*SP9D*^{pen} plants terminates after eight inflorescences, compared to five in the determinate M82 with an average of two leaves per sympodial unit (Carmel-Goren et al., 2003). Plants carrying the *S. pennellii* allele of *SP5G* presented developmentally delayed flowering (*i.e.* there were more leaves before the first inflorescence) and were significantly taller than M82 by means of more elongated internodes (Jones et al., 2007). In *SP5G*^{pen}/*SP5G*^{pen} plants, such as IL5-4, the number of nodes between inflorescences after the first inflorescence does not vary with respect to its isogenic control parent, M82. WELL was taller but flowering time was not affected. The WELL line like both *SP5G*^{pen}/*SP5G*^{pen} and *sp*/*sp* *SP9D*^{pen}/*SP9D*^{pen} plants, shows a semi-determinate growth habit. Determinate growth is present in MT (Marti et al., 2006) and most modern processing tomato varieties (Jones et al., 2007). The effect of determinacy on WUE of tomato or other species is not known. It appears that inherent in indeterminacy is a flexibility than enables individual plants to form few or many flowers, depending on the duration of favourable seasonal conditions (Stebbins, 1974). Natural selection should then favour indeterminacy in species adapted to semi-arid climates with great inter-seasonal variation in rainfall, like the wild relatives of tomato (Warnock, 1990; Warnock, 1991). In a field experiment comparing isogenic determinate and indeterminate soybean lines, evapotranspiration was similar but the determinate line produced 25% more grain by utilizing only 5.6% more water (Singh and Whitson, 1976). A more comprehensive study comparing field-grown determinate and indeterminate faba bean lines showed that high water availability promoted vegetative growth and decreased harvest index, the ratio of yield to total biomass produced (De

Costa et al., 1997). This effect was considerably more pronounced in the indeterminate plants, but these also showed a higher yield under water-limited growing conditions. The following chapters will examine how the growth habit difference between WELL and MT could affect WUE .

An effect of WELL is the more elongated internodes (Table 1). The same has been observed for introgression lines carrying *SP5G* allele of *S. pennellii*, although those lines did not show the progressively reduced number of internodes between inflorescences as WELL (Jones et al., 2007). Gibberellins lead to increased internode length and thus, increased height in tomato (Gray, 1957; Rappaport, 1957). Application of gibberellic acid (GA_3) to wild-type plants phenocopies the tall and slender constitutive-GA response mutant *procera* (Jupe et al., 1988). This, however, is accompanied by multiple developmental alterations such as paler leaves with lower margin indentation (Jones, 1987), exserted flower styli (Bukovac and Honma, 1967) and underdeveloped fruit (Rappaport, 1957), all of which are absent in WELL. Further, the number of internodes produced prior to first inflorescence is increased in *procer*a (9-10 internodes) compared to wild-type plants (6-7) (Jupe et al., 1988). This phenotypic effect was also reported for *sp*/*sp SP5G*^{pen}/*SP5G*^{pen} plants (Jones et al., 2007) but it is not present in WELL (Fig. 3). A second hormone class affecting internode length is ethylene. Antisense silencing of the ethylene receptor *LeETR1* produced plants with shorter internodes (Whitelaw et al., 2002). Significant elongation of internodes is an adaptive response of deepwater rice, which is thus adapted to flooding (Kende et al., 1998). It has recently been shown that two genes encoding ethylene response factors, *SNORKEL1* and

SNORKEL2, trigger the deepwater elongation response via gibberellin (Hattori et al., 2009).

Another possibility is that one of the known "dwarfing" loci in MT has been lost during the introgression process, or replaced by the *S. pennellii* allele. Among them are *SP*, *DWARF* and *MINIATURE*, which have been mapped to chromosomes 6, 5 and 11, respectively. This hypothesis will be addressed in Chapter 4 (Mapping of WELL).

2.5. Conclusion

An introgression line from *S. pennellii* in tomato was identified that has delayed wilting compared to its isogenic parent, the Micro-Tom cultivar, when both lines are subjected to water withdrawal in the same pot. This introgression line, named WELL, also shows semi-determinate growth habit, more elongated internodes and more erect leaf insertion angles. Two parallel approaches will be used to identify the functional basis of the WELL phenotype. The first one is physiological, through a series of experiments where the development, anatomy, gas exchange properties and water relations of WELL and MT are compared to test the various hypotheses put forward to explain WELL's delayed wilting. The second approach is genetic, to map the *S. pennellii* introgression on the MT genome, examining the inheritance of the associated phenotypes, and determining whether these reflect pleiotropic effects of a single genetic locus or the action of several clustered genes segregating together.

The questions addressed in the remainder of this thesis for the functional characterisation of the WELL introgression are:

- Do WELL plants have improved WUE?
- What are the causes of the delayed wilting of WELL under limited water supply?

These questions will be examined in Chapter 3.

- Do the various phenotypes of WELL reflect pleiotropy or the action of several genes?
- Where does the WELL introgression map in the tomato genome?
- Can recombinants be recovered where linkage of the various phenotypes of WELL is broken?

These questions are addressed on Chapter 4.

Chapter 3 – Physiological characterisation of WELL

3.5. Conclusion

3.1. Introduction

An introgression line from *S. pennellii* was created in tomato cv. Micro-Tom (MT) by screening leaves for the propensity to wilt under limited water supply. This line, WELL, is characterised by a significant delay in leaf wilting upon suspension of watering, as well as increased height, semi-determinate growth habit, and pink fruits. The physiological basis for the delayed wilting is explored in this chapter. A first hypothesis is that alterations in leaf development lead to a reduced leaf area in WELL. This would cause a lower transpirational demand and thus, all other parameters being equal, lead to a delayed wilting compared to MT in drought conditions. Comparison of plants grown in the same pot during the screening process, however, has already eliminated leaf area effects on time to wilting. Two plants in a single pot will reach the threshold of water depletion in the pot at the same time, as determined by the combined leaf area and conductance of the two plants. After that, depletion of water depends on water loss per unit leaf area, provided that hydraulic conductances are the same. A second possibility is that modified stomatal development and/or dynamics could effect a lower stomatal conductance to water vapour. This hypothesis is tested in the present chapter.

I also explored whether WELL plants have higher water-use efficiency (WUE), measured either as the amount of plant dry matter gained per unit of water lost or as leaf transpiration efficiency (*TE*), defined as the ratio between photosynthetic CO_2 assimilation rate (*A*) and

transpiration rate (*E*). The delayed wilting of WELL is probably a physical expression of drought resistance, and more specifically, dehydration avoidance through limited plant water loss under conditions of low soil water potential (Levitt, 1972). Increased efficiency of water use could be a physiological consequence of improved drought resistance, caused by reduced stomatal conductance. WELL wilts after MT, while continuing to produce leaves, suggesting that it may have the capacity to produce more biomass using less water. Increased WUE has been observed for the drought-resistant wild species *S. pennellii* (Martin and Thorstenson, 1988; Rick, 1973; Yu, 1972). As discussed in Chapter 2, a number of genetic as well as environmental factors could explain the delayed wilting of WELL, and possibly increased WUE. A series of experiments were therefore conducted under various growth conditions chosen to create variations in parameters that drive transpiration and maintenance of cellular turgor, as well as the demand and supply for water and carbon.

3.2. Methods

3.2.1. Plant material

Solanum lycopersicum L. cv. Micro-Tom (MT) was compared with the WELL introgression line, described in Chapter 3, and an indeterminate line of MT tomato, which was generated as follows. The determinate growth habit of the MT tomato cultivar is at in least in part due to a single-base substitution at position 227 of the coding sequence in

the *SELF-PRUNING* (*SP*) gene (Marti et al., 2006; Pnueli et al., 1998). An indeterminate MT sp^+/sp^+ line was created by introgressing the functional allele of *SP* into MT from the Moneymaker cultivar. The process of introgression consisted of a series of crosses and backcrosses (BC) using MT as the recurrent parent. In the first cross, pollen was collected from Moneymaker plants and used to fertilize emasculated MT flowers. The offspring of the first cross (F_1) were self-pollinated to produce a recombinant F_2 generation from which plants were selected for small size and indeterminate growth habit. Indeterminate plants are easily recognised visually as they produce an unrestricted number of sympodial units, resulting in a vine-like plant structure. The selected individuals were backcrossed with MT up to the sixth generation (BC_6) and then selfed. BC_6F_2 homozygous sp^+/sp^+ plants were identified through segregation analysis when their offspring were 100% indeterminate. At this point these plants can be theoretically considered near-isogenic to the recurrent parent (Reid, 1993; Stam and Zeven, 1981).

3.2.2. Growth conditions

Six experiments were performed. The setup and conditions for each are described in Table 1. Growth conditions are also specified where relevant in the text. Common methods for all experiments are detailed in the following sections.

Table 1. Summary of conditions for the experiments presented in this chapter.

Exp ID	Aim/Measurements	Soil type	Pot vol L	Age of plants at harvest (d.a.g.)	Harvest before/ after flowering	Replicates per genotype	Environment	Temp (day/night) °C	Irradiance µmol photons m^{-2} s^{-1} PAR	Photoperiod (hours of light)	Watering regime
1	Determine WUE	Sand	3	30 and 50	B/A	6 and 4	Glasshouse	26/20	500-800	10	Every 2nd day
2	Test effect of indeterminacy	Sand	1.7	60	A	4	Glasshouse	26/20	500-800	10.5	Every 2nd day
3	Stomatal conductance	Sand	1.7	-	A	5	Glasshouse	26/20	600-1000	11	Every 2nd day
4	WUE	Sand	1.7	30, 45 and 51	B/A/A	8	Growth chamber	25/20	600	12	Every 2nd day
5	Response to drought	Soil mix	0.15	45	B	5	Glasshouse	25/20	500-800	11.5	Capillarity
6	Stomatal conductance under drought	Sand	1.7	-	A	5	Growth chamber	25/20	350	12	Daily

3.2.3. Gravimetric measurement of whole plant WUE

Gravimetric long-term WUE was determined in Experiments 1, 2 and 4. Seedlings were transplanted to pots when the first pair of leaves was visible, and after allowing 2 days for plant acclimation and root establishment, pots were flushed with full-strength modified Hoagland's nutrient solution (Hoagland and Arnon, 1938). When dripping stopped, the pots were covered with plastic foil tied with a rubber band and weighed. This weight was recorded as the target 'initial weight' for subsequent watering. Every 24h, at the end of the day, pot weight was determined and recorded. Pot weight was brought back to the initial 'target' weight through addition of nutrient solution. Control pots with no plants were included and weighed to calculate the amount of water lost through soil evaporation. Plant shoots and roots were harvested and dried at the end of the experiment and WUE was determined as the ratio between plant dry mass and amount of water transpired during the same period:

Transpired water for pot n = water lost by pot n (g) – average water lost by control pots without plants (g)

In some experiments (Experiment 1) where water loss was monitored from the day seedlings were transplanted, plant mass at this time was assumed to be negligible compared to that at the end of the experiment when plants were harvested, so that WUE was calculated as:

Plant dry mass (g) on final day / Transpired water (kg) between transplanting and final day

In other experiments where WUE was determined for specific periods (t_0-t_1) at various stages of the plant cycle, a batch of plants was harvested on the first day water loss was monitored (t_0) and WUE over the period from t_0 to t_1 was calculated as:

WUE_{t0-t1} = [Plant dry mass t_1 (g) – Plant dry mass t_0 (g)]/ Water transpired between t_0 and t_1

Student's *t*-tests for statistical significance were performed with the online software GraphPad (http://www.graphpad.com/quickcalcs/index.cfm). ANOVA and Tukey's HSD test were performed using the online tool VassarStats (http://faculty.vassar.edu/lowry//anova1u.html).

3.2.4. Gas exchange measurements

CO_2 and water vapour fluxes in and out of leaves were measured using a LI-6400 portable photosynthesis system (Li-Cor, Nebraska, USA). All measurements were performed on intact plants in the glasshouse/growth chamber on a fully expanded leaf of the same rank across all plants

compared. When necessary, area corrections were made in the calculations for leaves not covering the whole 6 cm^2 of the chamber. Measurements were taken under steady-state after equilibration, to measure stomatal conductance (g_s). Photon flux density (1500 μmol m^{-2} s^{-1}, from the LED source), leaf temperature (25 ± 0.5°C), leaf-to-air vapour pressure difference 17.5 ± 2.5 mbar) and air flow rate into the chamber (500 μmol s^{-1}) were all held constant while ambient CO_2 concentration was set at 400 ppm (CO_2 injected from a cartridge). The parameters describing photosynthetic capacity (V_{cmax} and J, maximum Rubisco carboxylation rate and electron transport rate, respectively) were estimated using the fitting utility by Sharkey et al. (2007), and theoretical assumptions therein, available online at http://www. blackwellpublishing.com/ plantsci/pcecalculation/

Gas exchange data analyses and plots were done using Microsoft Excel 2003 and Python 2.7.1.

3.2.5. Determination of carbon isotope discrimination and its relationship to WUE

Carbon isotope discrimination is another way of assessing WUE. Oven-dried plant material consisting of either total above ground matter or a

reference leaf (indicated in the text) was ground using a rotary mill. Samples were analyzed for $^{13}C/^{12}C$ ratio with an Isochrom mass spectrometer (Micromass, Manchester, UK) operating in continuous flow mode. The absolute isotopic composition of a sample is not easy to determine, instead the mass spectrometer measures the isotopic composition with respect to a standard. The international reference standard for carbon isotope ratios is limestone from the Pee Dee Formation (South Carolina, USA) derived from the Cretaceous marine fossil *Belemnitella americana* and thus abbreviated PDB. The isotopic composition, $\delta\,^{13}C$, of atmospheric CO_2 was assumed to be -8 ‰ (read 'per mil', parts per thousand, the minus sign indicates that there is less ^{13}C than in the PDB standard). The $\delta^{13}C$ values for the samples were then converted to carbon isotopic discrimination values, $\Delta^{13}C$:

$$\Delta^{13}C = (\delta_a - \delta_p)/(1 + \delta_p) \qquad \textit{Eqn 1}$$

where δ_a is the $\delta^{13}C$ of atmospheric CO_2 and δ_p is the $\delta^{13}C$ of the plant material.

$\Delta^{13}C$ is a surrogate measure of transpiration efficiency (*TE*, also known as instantaneous water-use efficiency), the ratio of CO_2 assimilation rate (*A*) to transpiration rate (*E*) because both parameters, $\Delta^{13}C$ and *TE*, are

linked to a third leaf gas exchange parameter, p_i/p_a, which is the ratio between internal and ambient CO_2 partial pressure (Farquhar and Sharkey, 1982). About 1% of carbon in atmospheric air is ^{13}C. During photosynthesis, the lighter isotope ^{12}C is preferentially fixed by plants, hence the expression 'isotopic discrimination'. The two main sources of discrimination against ^{13}C are slower diffusion through stomata and lower reactivity with Rubisco (Farquhar et al., 1989). In its simplest form, the equation describing the relationship between carbon isotopic discrimination and pi/pa is given by:

$$\Delta = a + (b - a)\, p_i / p_a \qquad \textit{Eqn 2}$$

where a is the isotopic fractionation during diffusion through the stomata (≈4.4‰, O'Leary, 1981) and b is the effective fractionation by Rubisco (≈27‰, depending on assumptions on mesophyll and cell wall conductance; Farquhar, 1984).

At the leaf level, instantaneous transpiration efficiency (*TE*) is the ratio between CO_2 assimilation rate (A) and transpiration rate (E):

$$TE = \frac{A}{E} = \frac{g(p_a - p_i)}{1.6 g \nu} = \frac{p_a(1 - p_i / p_a)}{1.6\nu} \qquad \textit{Eqn 3}$$

where g is the conductance of the leaf to diffusion of CO_2, v is leaf-to-air vapour pressure difference and 1.6 is the ratio of the diffusivities of water vapour and CO_2 in air. It can be seen from equation 3 that lower values of p_i / p_a will result, in the simplest case, in an increased *TE*. Lower p_i / p_a ratios can come about through lower g_s, higher photosynthetic capacity, or a combination of both (Farquhar et al., 1989).

Equations 2 and 3 can be combined to relate Δ and *TE*:

$$TE = \frac{p_a}{1.6v} \cdot \frac{b - \Delta}{b - a} \qquad \textit{Eqn 4}$$

whereby Δ and *TE* are negatively related. This relationship has been confirmed experimentally in several species (Farquhar and Richards, 1984; Hubick and Farquhar, 1989; Hubick et al., 1986; Virgona et al., 1990), including tomato (Martin and Thorstenson, 1988). The demonstration of a strong heritability for carbon isotope discrimination (Condon and Richards, 1992; Rebetzke et al., 2002a), has made it a widely used tool for studies of WUE and breeding programs towards its improvement in crops (Condon et al., 2004; Martin et al., 1999)

3.2.6. Leaf anatomy

Mature, fully-expanded and well-exposed leaflets (typically from leaves 4-6 counted from the base of the primary stem) were sampled and cleared in 100% methanol at ambient temperature for 48h. When leaves had lost all the chlorophyll and turned white, they were transferred to scintillation vials filled with 95% lactic acid and incubated on a hot plate at ~100°C for approximately 30 minutes. Cleared leaves were mounted on glass slides and examined by light microscopy (Nikon Optiphot light microscope). Images were analysed using ImageJ 1.42q (NIH, Bethesda, MA, USA) for determination of stomatal density and analyses of cell sizes in leaf cross-sections. Stomata were counted on 6 different fields of view per leaf at 40x magnification.

For analyses of mesophyll anatomy, 5 × 3 mm samples were cut from the centre of a fully-expanded well-exposed leaflet and fixed in a 2.5% glutaraldehyde and 4% formaldehyde solution in 0.1M PBS for 2 hours (Karnovsky, 1965). Samples were then washed in distilled water and dehydrated in an ethanol series [50, 60, 70, 80, 90 and 100% (v/v)]. Samples were infiltrated in a series of 2:1, 1:1 and 1:2 100% Acetone : Epon Araldite (30 minutes per change) and finally 3 changes of 2 hours each in pure Epon Araldite. Embedding was done in fresh resin and cured overnight at 60°C.

Five-micrometer thick cross-sections were cut using a rotating microtome with a glass knife (Reichert Ultracut). The sections were mounted in water on glass slides and stained with 0.05% (v/v) toluidine blue in phosphate buffer and citric acid (Sakai, 1973). Histological sections were observed using an optic microscope (Nikon Optiphot) connected to an image capture system (SPOT Insight 12 bit camera) and analyzed for leaf thickness and palisade and spongy mesophyll cell sizes and numbers. This was done on leaves of 4 different plants per genotype, in the section between the primary leaf vein and the first secondary vein to the side.

3.2.7. Water loss from detached leaves

Water loss from detached leaves is a useful indicator of resistance to desiccation controlled by processes in the leaf as opposed to systemic effects of the whole plant. Leaflets from fully-expanded leaves were cut at the base of the petiolule with a sharp razor blade. Leaflet area was measured using a LI-3050A Conveyer Leaf Area Meter (Li-Cor, Nebraska, USA). Leaflets were rehydrated in distilled water for 2 hours in the dark and then weighed to 0.001 g with an analytical balance to determine their weight at full turgor. They were next left at room temperature at 25°C on a bench, abaxial (lower) side facing down, and in the dark for 40 minutes, and weighed every 5 minutes. Water loss per unit leaf area was determined for each 5 min interval and plotted. Samples were then oven-dried at 70°C for 24 hours and dry weight determined.

3.2.8. Relative water content (RWC)

RWC is an indicator of the water status of a plant, since it expresses the absolute amount of water which plants require to reach artificial saturation (Slatyer, 1967). RWC is calculated as the percentage in water content at a given time as related to the water content at full turgor:

RWC = (FW-DW)/(TW-DW)

where FW: fresh weight (weight at the time of leaf detachment); DW: dry weight; TW: turgid weight (after rehydration).

3.2.9. Leaf water potential measurements

Pre-dawn leaf water potential was estimated using a pressure bomb (PWSC Model 3000, Soil Moisture Equipment Corp., California, USA). Measurements were performed starting 2h before the growth chamber lights went on, *i.e.* on non-transpiring leaves. Terminal leaflets of well-exposed leaves were bagged in Whirl-Pak bags (Enasco, USA) and quickly cut from the base of the petiolule with a sharp razor blade. The detached leaflet was quickly inserted in the chamber and pressurized until the cut surface of the petiolule became wet and shiny. The balancing pressure was recorded at this point.

3.3. Results

3.3.1. WELL has a higher WUE than MT after flowering

In the first experiment MT and WELL plants were grown in well-watered conditions in the glasshouse (conditions detailed in Appendix 3A). The aims were: 1) to measure the water transpired during the post-flowering period where the difference in wilting between the two genotypes was seen (Chapter 2); 2) investigate the possible role of differences in leaf area in determining different rates of soil water depletion and hence wilting; 3) concurrently quantify water transpired and biomass accumulated to calculate WUE. MT and WELL plants were grown in 3L pots and watered to 'field capacity' with nutrient solution every second day. Two harvests were performed, before and after flowering of both genotypes, to account for the effects of different plant architecture between MT and WELL, which only becomes evident after flowers open. Leaf area and plant dry mass were determined at each harvest and $\Delta^{13}C$ was measured for each genotype in a pool of aboveground dry tissue. Gas exchange was measured at the beginning of fruit set in well exposed mature leaves of intact plants.

There was no significant difference between genotypes in whole plant fresh weight (data not shown) but dry weight was 10% higher in MT (Table 2). The main driver of this difference was a significant increase in leaf dry

mass (32%). Total water transpired between germination and the onset of flowering at day 30 was similar for both MT and WELL (Table 3). There was no difference in carbon isotope discrimination ($\Delta^{13}C$) measured in aboveground tissues (Table 2).

Table 2. Experiment 1. Growh parameters for MT and WELL measured 30 days after germination, at the onset of flowering. Mean ± s.e.m. (n=6). *p*-values calculated with a *t*-test: ** indicates significant differences at $p<0.001$

	MT	WELL	*p*
Dry mass (g)			
Leaves	0.50 ± 0.01	0.40 ± 0.02	0.0004 **
Stem	0.10 ± 0.01	0.13 ± 0.01	0.060
Root	0.17 ± 0.02	0.16 ± 0.02	0.73
Total	0.77 ± 0.03	0.69 ± 0.04	0.14
$\Delta^{13}C$ ‰	22.15 ± 0.17	22.05 ± 0.06	0.71
Leaf area (cm^2)	163 ± 4	111 ± 6	0.0001 **
SLA (cm^2/g)	326 ± 4	324 ± 22	0.92

A second batch of plants from Experiment 1 was sampled 50 days after germination, at the beginning of fruit set. The amount of water transpired was still similar for both genotypes, but WELL plants had at this point outgrown MT by around 25%, as measured by total dry mass (Table 3). The difference in dry weight was largest for leaves but also significant for stems, while root dry weight was similar (Table 3). Due to increased biomass accumulation, WUE over the 20-day period after flowering (days 30-50) was

higher in WELL (Table 3). This was reflected in the ranking of leaf $\Delta^{13}C$ values, which were about 1 per mil lower in WELL than MT (Table 3).

Leaf area was also higher in WELL, but non significantly and comparatively less than leaf dry-weight, *i.e.* the amount of C per unit leaf area increased, indicating that leaves were thicker and/or denser. Specific leaf area (SLA, the amount of leaf area per unit dry mass of leaf) was significantly lower in WELL compared to MT, whereas at the previous sampling there was no difference between the two lines (Table 2).

Table 3. Experiment 1. Growth parameters for MT and WELL values measured on day 50, before the beginning of fruit set. Water transpired measured between days 30 and 50 after germination. Mean ± s.e.m. (n=4). *p*-values calculated with a *t*-test: * and ** indicate significant differences at $p<0.05$ and $p<0.001$ respectively.

	MT	WELL	*p*
Dry mass (g)			
Leaf	0.77 ± 0.06	1.08 ± 0.01	0.037 *
Stem	0.59 ± 0.06	0.78 ± 0.04	0.042 *
Root	0.34 ± 0.09	0.42 ± 0.06	0.45
Total	1.69 ± 0.11	2.28 ± 0.19	0.035 *
Water transpired (d30 – d50) (g)	636 ± 19	643 ± 16	0.79
WUE (d30 – d50) (g/kg)	2.67 ± 0.09	3.66 ± 0.31	0.020 *
$\Delta^{13}C$ ‰	22.05 ± 0.12	21.19 ± 0.19	0.0004 **
Leaf area (cm^2)	233 ± 15	266 ± 19	0.23
SLA (cm^2/g)	304 ± 15	247 ± 13	0.0001 **

Leaflet cross-sections were examined by light microscopy to see if there were anatomical differences that could explain the lower SLA in WELL (Appendix 3B). Both the length and area of palisade mesophyll cells were greater in WELL than in MT (Table 4). There was no difference in leaf thickness between genotypes (Table 4), so the differences in cell size could contribute to a difference in SLA, although this needs to be confirmed as cell numbers nor cell wall thickness were not measured.

Table 4. Experiment 1. Comparison of leaf blade anatomy of MT and WELL plants. Leaflets sampled 50 days after germination. Mean ± s.e.m. (n=4). *p*-values calculated with a *t*-test: * indicates significant differences at $p<0.05$

	MT	WELL	*p*
Palisade cell length (μm)	36.6 ± 1.9	46.1 ± 0.9	0.0044 *
Palisade cell width (μm)	9.1 ± 0.5	8.8 ± 0.3	0.69
Palisade cell area (μm^2)	29.0 ± 0.8	33.8 ± 0.9	0.0026 *
Leaf thickness (μm)	104.6 ± 2.0	101.8 ± 1.6	0.30

Stomatal and trichome densities were measured in fully expanded mature leaves of both genotypes to determine whether anatomical differences could be influencing carbon fixation and water loss. There was no significant difference in stomatal density on the adaxial side, but an increase was seen in the abaxial side for WELL (Fig 1a). Trichome densities were similar for both genotypes on either face of the leaf (Fig 1b). This suggested a lower stomatal aperture in WELL since similar amounts of water were

transpired by the two lines through similar total leaf areas. Leaf gas exchange measurements were therefore performed to clarify this issue.

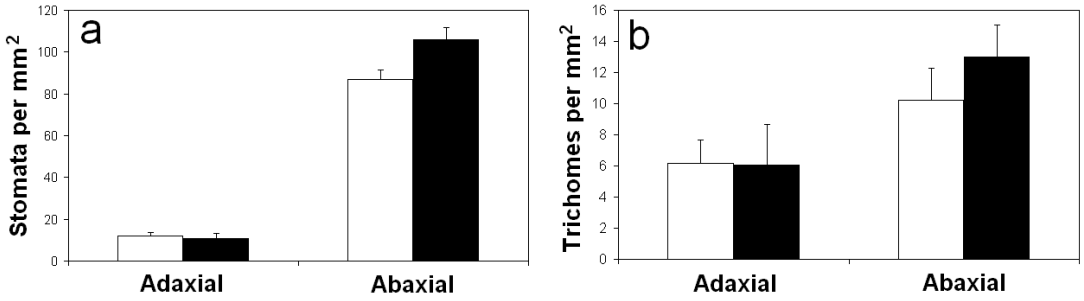

Figure 1. Experiment 1. (a) Stomatal and (b) trichome density in mature and fully-expanded leaves of MT (open bars) and WELL (full bars). Bars represent s.e.m. (n=4 leaves).

Gas exchange was measured at day 60 after germination to assess the cause for the higher WUE in WELL leaves after the onset of flowering. The response of CO_2 assimilation rate to varying intercellular CO_2 concentrations provides a way to distinguish between stomatal and biochemical limitations to photosynthesis. Under ambient CO_2 concentration (400ppm) the difference in *TE* appeared mostly due to a large difference in g_s, which was 44% lower in WELL, resulting in a 13% higher *TE* (Table 4). No significant difference was observed between MT and WELL in apparent Rubisco activity (V_{cmax}) or maximum rate of electron transport (J_{max}) used in the regeneration of ribulose-1,5-*bis*phosphate (Table 5).

Table 5. Experiment 1. Gas exchange parameters for MT and WELL measured in 60-day-old plants. Mean ± s.e.m. (n=4). *p*-value calculated with a *t* test: * and ** indicate significant differences at $p<0.05$ and $p<0.001$ respectively.

	MT	WELL	*p*
A (μmol CO_2 m^{-2} s^{-1})	14.04 ± 0.51	10.50 ± 0.68	0.0007 **
g_s (mol H_2O m^{-2} s^{-1})	0.23 ± 0.01	0.13 ± 0.01	0.0002 **
E (mmol H_2O m^{-2} s^{-1})	3.68 ± 0.18	2.41 ± 0.19	0.0021 *
TE (A/E) (μmol CO_2 per mmol H_2O)	3.84 ± 0.12	4.40 ± 0.16	0.032 *
V_{cmax} (μmol m^{-2} s^{-1})	90.3 ± 3.7	89.7 ± 10.7.	0.95
J_{max} (μmol m^{-2} s^{-1})	127.8 ± 6.3	121.8 ± 14.4	0.71

The results show that WELL has a higher *TE* at the leaf level, concurring with a higher long-term WUE (see Table 3), both of which appear to develop only after the onset of flowering. The difference in *TE* was driven by a difference in *E* and stomatal conductance, while photosynthetic capacity was similar for the two lines. Since WELL has a semi-determinate growth habit, it was hypothesized that growth habit could play a role in its lower stomatal conductance. Although leaf areas were similar at day 50, there may have been a significant enhancement of leaf area production past that point in the semi-determinate WELL compared to the determinate MT, so that by the time gas exchange was measured at day 60, the increased transpirational area may have led to transient water stress in WELL, inducing stomatal closure. The next experiment was conducted to assess the effect of determinacy on WUE.

3.3.2. Does growth habit affect WUE?

Experiment 2 was carried out to compare WUE between MT and MT *sp*$^{+}$*/sp*$^{+}$, an isogenic line containing the wild-type (*i.e.* functional) allele of *self-pruning*, which produces an indeterminate growth habit. Plants were grown in 1.7 L pots and at the four leaf stage the pots were covered and water loss monitored over the next 35 days. Plants were then harvested and leaf area, dry mass and total water loss over the whole period were determined. $\Delta^{13}C$ was measured in a reference fully-exposed mature leaf for each replicate plant.

No macroscopic difference was evident between genotypes before flowering (Fig 2). Water loss increased as plants grew and expanded their leaves but was similar between genotypes (Fig 3).

Figure 2. Experiment 2. Representative 30-day old MT (left) and MT *sp*$^{+}$*/sp*$^{+}$ (right) individuals.

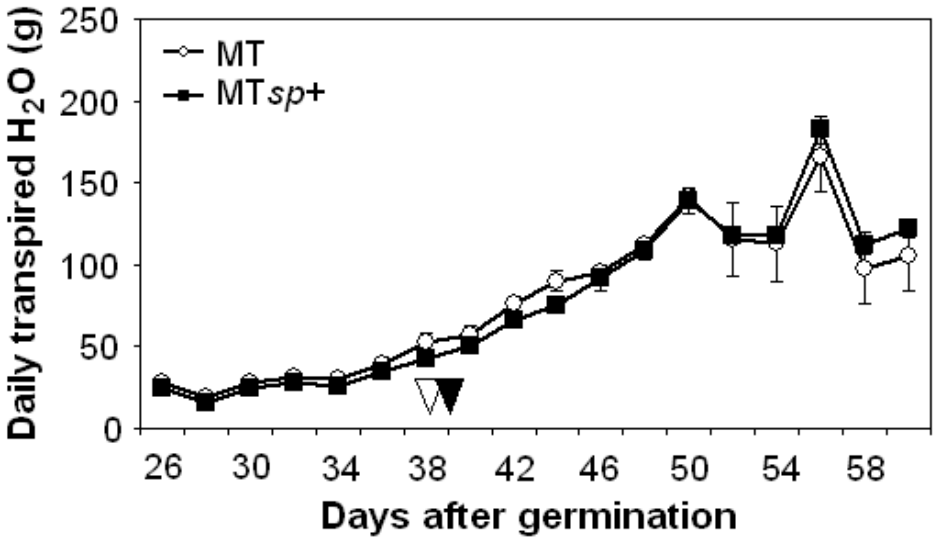

Figure 3. Experiment 2. Water loss by transpiration in MT and MT *sp*⁺/*sp*⁺Arrows mark flowering time. Bars represent s.e.m. (n=4).

Even with similar total water loss between genotypes, the transpiration rates per unit leaf area between the two genotypes could be different, depending on the plants' leaf areas. The area of individual leaves varied with position between the two genotypes, and the determinate MT stopped producing primary leaves at the onset of flowering, whereas MT *sp*⁺/*sp*⁺ kept producing primary leaves throughout the whole 35-day period (Fig 4a). The total values of leaf area were, however, remarkably similar (Fig 4b) due to a larger contribution in MT from leaves of axillary branches derived from axillary meristems (Fig 4b).

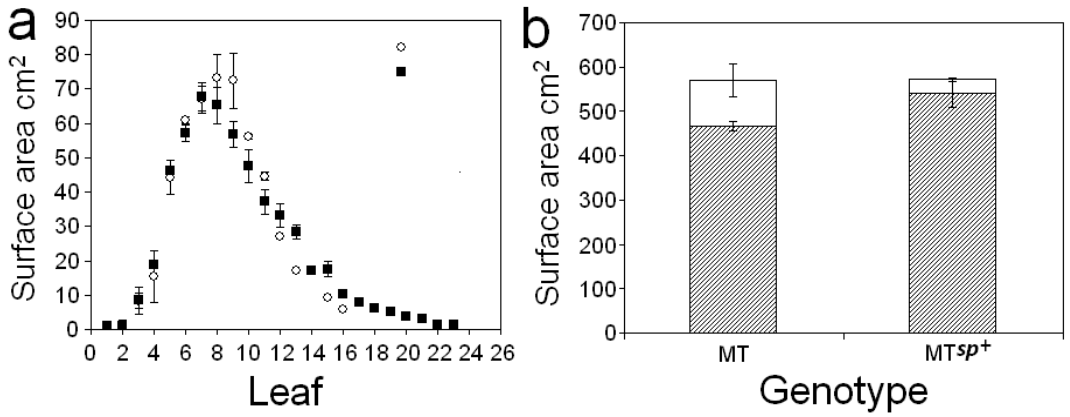

Figure 4. Experiment 2. (a) Surface area of successive leaves with leaf position from bottom to top in MT (circles) and MT *sp*⁺/*sp*⁺ (squares). (b) Plant total leaf area showing the contribution of primary (shaded) and axillary (white) leaves. Bars represent s.e.m. (n=4).

A low number of plant replicates was available for this experiment so no plant was harvested for determination of initial dry-weight at the time gravimetric measurements of water loss were started, 25 days after germination. Based on the plant sizes at this time, which were similar for both genotypes, and on dry-weights measured for MT in earlier experiments under similar conditions, the seedlings' dry weight on day 35 was here assumed to be 1 g for both genotypes and this value was used as initial dry-weight to calculate biomass accumulated between day 35 and day 60, when all plants were harvested. Total dry mass was not significantly different between genotypes at that end point, nor was the estimated whole plant WUE over the 35 days from day 25 to day 60 (Table 6). Under the conditions used in this experiment, the difference between determinate and indeterminate growth habit between the two lines caused by allelic variation at the *S* locus did not induce -nor was accompanied by- a difference in total transpiring leaf area. This does not rule out, however, that other genes which are causing the difference in determinacy observed between WELL and MT

may affect WUE, through effects on leaf area expansion and plant architecture, and possibly directly through, for instance, hormonal effects on stomatal dynamics.

Table 6. Experiment 2. Water-use efficiency (WUE) in MT and MT *sp*⁺/*sp*⁺ measured 60 days after germination. Mean ± s.e.m. (n=4). *p*-values calculated with a *t* test.

	MT	MT *sp*⁺/*sp*⁺	*p*
Water transpired (d25-d60) (kg)	1.39 ± 0.14	1.37 ± 0.04	0.91
Dry mass (d25-d60) (g)	3.90 ± 0.23	3.66 ± 0.25	0.49
WUE (g/kg)	2.80 ± 0.26	2.67 ± 0.10	0.30

3.3.3. Stomatal conductance is lower in the introgression line at the same soil water potential as MT

The results of Experiment 1 suggested that the lower g_s of WELL compared to MT after flowering is a consequence of a different rate of water extraction from the soil. Different rates of soil water depletion can occur through differences in transpiring leaf area, all other parameters being equal. However, during the introgression process, MT and the plants derived from the cross to *S. pennellii* of successive generations, were grown paired in a single pot, thus ruling out the effect of transpiring area on time to wilting, assuming similar hydraulic resistances. As selection on wilting was not applied at all steps and is somewhat subjective, experiment 3 was designed to measure g_s of paired plants i.e. under a similar soil water potential for both

genotypes. MT and WELL plants were grown paired in a single pot filled with sand and watered with nutrient solution every second day. Gas exchange measurements were performed after flowering in well exposed mature leaves of intact plants.

The results show that g_s in WELL was reduced by 60% compared to MT throughout the whole measurement period (Fig 5b), suggesting that 1) WELL plants may have experienced water stress leading to reduce stomatal conductance, 2) stomatal sensitivity to that water stress was enhanced in WELL compared to MT. It should be noted, however, that the data indicate lower stomatal conductance in WELL leaves also under well-watered conditions.

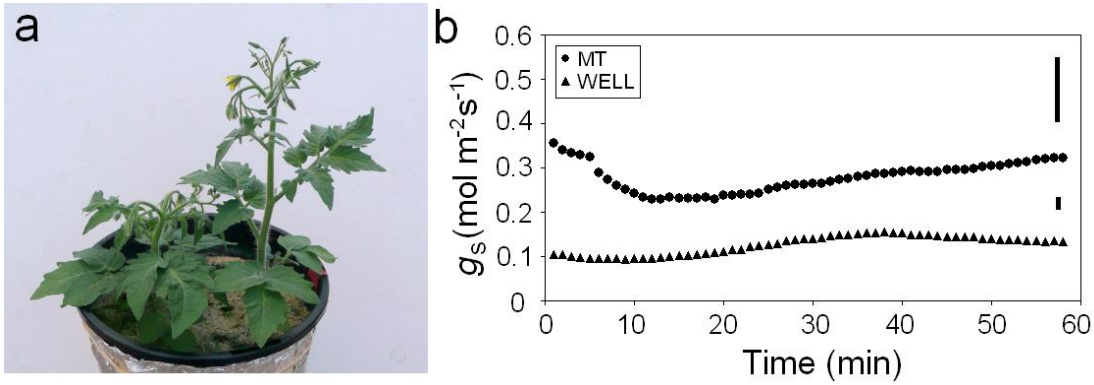

Figure 5. Experiment 3. (a) Representative 40-day-old plants of Micro-Tom (left) and WELL (right). (b) Stomatal conductance to water vapour (g_s) in Micro-Tom and WELL. Bars represent s.e.m. for MT (top) and WELL (bottom), n=4. Measurements were performed two days after the plants were last watered.

3.3.4. WELL has lower stomatal conductance than MT under drought

In order to more closely examine stomatal conductances and its responses to water stress, another experiment (Experiment 4) was performed , where MT and WELL plants were flushed daily with nutrient solution until flowering (day 45) and then split into two batches, one where the same watering regime was continued , the other where watering was withheld for 6 days. Gravimetric WUE measurements were carried out over this 6 day period and mature leaves were sampled on the final day for $\Delta^{13}C$ determinations. Gas exchange was measured on day 5 (beginning of fruit set), in well exposed mature leaves of intact plants.

The results confirmed the observation made in Experiment 1 that under well-watered conditions MT and WELL had similar WUE before flowering (days 30-45, Table 7). After the onset of flowering (days 45-51) however, WUE was increased in WELL. This increase, however, was statistically significant in the treatment where water had been suspended but not in the control, as measured directly by gravimetry, or indirectly by $\Delta^{13}C$ (Table 7).

Table 7. Experiment 4. MT and WELL values for long-term water-use efficiency (WUE, g plant dry mass per kg water transpired), over two successive periods, before and after flowering (30-45 and 45-51 days after germination, respectively). Mean ± s.e.m. (n=8) *p*-values calculated with a *t*-test: * indicates significant differences at $p<0.05$

	MT	WELL	*p*
Pre-flowering			
WUE d30-d45	5.42 ± 0.28	5.66 ± 0.14	0.46
Post-flowering control			
WUE d45-d51	4.59 ± 1.33	7.81 ± 0.75	0.068
$\Delta^{13}C$	20.43 ± 0.43	20.09 ± 0.31	0.53
Post-flowering drought			
WUE d45-d51	4.10 ± 2.07	9.91 ± 0.48	0.025 *
$\Delta^{13}C$	21.17 ± 0.17	18.95 ± 0.71	0.016 *

Gas exchange was measured five days after the suspension of watering in control and water-stressed plants. In the control treatment, there was no difference between genotypes in photosynthetic rate or transpiration rate (Table 8). Under drought, however, WELL showed considerable reductions of 50% in *A* and 65% in g_s, compared to its own well-watered controls (Table 8), which was not the case of MT leaves. Consequently, A and g_s were statistically significantly lower when compared to those in water-stressed MT plants (Table 8).

Table 8. Experiment 4. MT and WELL values of gas exchange parameters measured 51 days after germination. Mean ± s.e.m. (n=4). * indicates significant differences at p <0.05 with Tukey's HSD test

	MT		WELL	
	Control	*Drought*	*Control*	*Drought*
A (μmol CO_2 m^{-2} s^{-1})	12.38 ± 0.90	11.30 ± 1.0	13.78 ± 0.66	7.08 * ± 2.22
g_s (mol H_2O m^{-2} s^{-1})	0.15 ± 0.02	0. 14 ± 0.02	0.17 ± 0.03	0.06 * ± 0.03
TE (A/E) (μmol CO_2 $mmol^{-1}$ H_2O)	4.33 ± 0.26	4.35 ± 0.63	4.34 ± 0.37	5.25 ± 0.51

The results presented in this section showed that the stomatal response of WELL to water limitation in the soil is enhanced compared to MT. This difference seems, nevertheless, to be expressed only after the onset of flowering. Another experiment (Experiment 5) was conducted to test the effects of water withdrawal imposed at early stages of plant development, when the plants of both genotypes are similar in structure and size.

3.3.5. WELL improves maintenance of turgor even when drought is imposed before flowering

The aim of Experiment 5 was to assess whether the stomatal response under drought, which was previously studied in plants after flowering (Experiments 1 and 4), was also different before flowering and thus, before the onset of the semi-determinate growth habit in WELL. A second aim was to test whether long-term (weeks instead of days) drought tolerance was also improved in WELL. Seedlings were transplanted into small pots (150 mL) at

the two leaf stage and split into two treatments: well-watered (sub-irrigation from trays) and drought (no watering). After two weeks, when they appeared completely wilted, the plants of the drought treatment were re-watered for 48 hours and then water was removed again. Plants were harvested 48 days after germination.

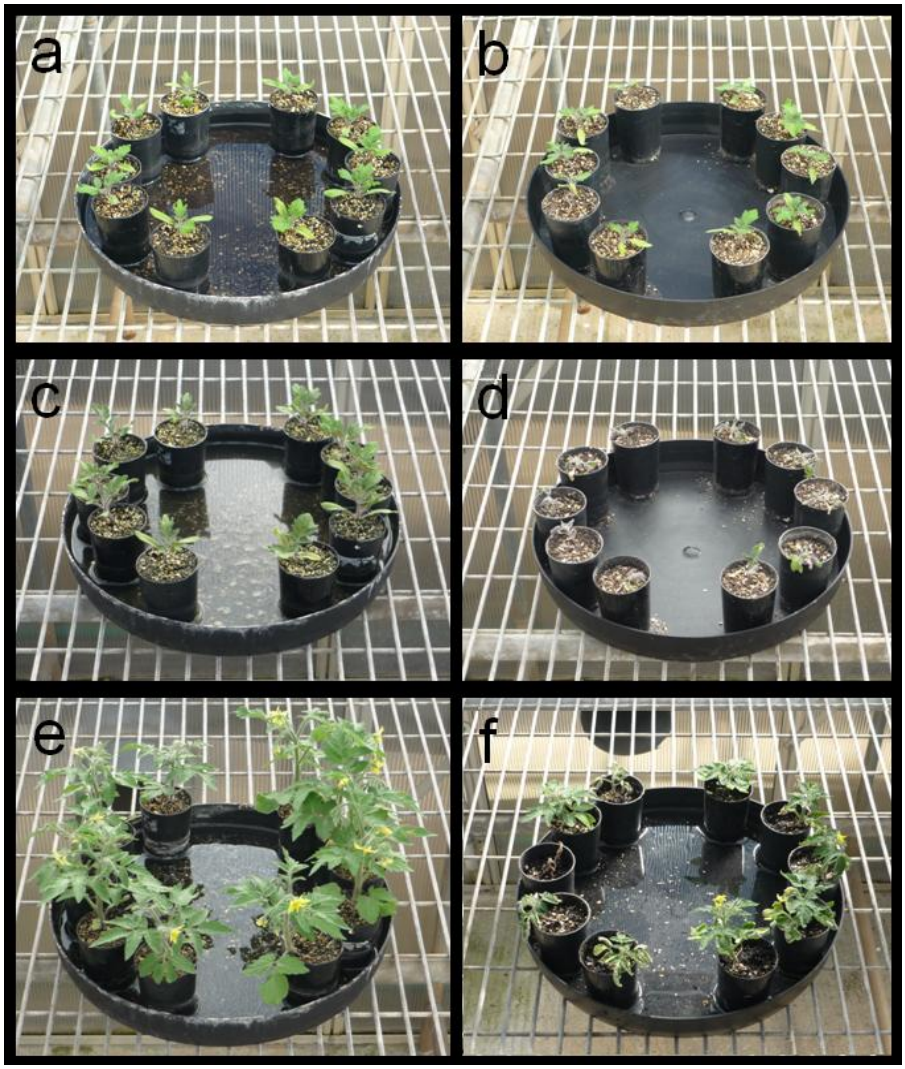

Figure 6. Experiment 5. Drought and recovery in MT and WELL. In each tray: five replicates of MT in individual pots (pots on the left in the tray) and five plants of WELL (pots on the right in the tray). Top two panels: 18-day old plants (a) Control; (b) Water withheld for 4 days. Center panels: 26-day old plants (c) Control and (d) Drought. Plants were rewatered that day for 2 days. Bottom: 45-day old plants (e) Control; (f) Drought, after re-watering overnight. Plants were harvested on that day.

By the second week of drought, plants of both genotypes were very wilty and had a much reduced size (Fig 6d compared to 6c). After the second period of water deprivation (14 days) followed by re-watering for 1 day, all leaves of the 5 WELL plants recovered turgor within 1 day, compared to only one plant out of the 5 MT plants (Fig 6f). Interestingly, three WELL plants caught up phenologically with the well-watered ones and had open flowers at the end of the experiment. None of the MT plants in the drought treatment had flowered after rewatering at the end of the experiment (Fig 6f).

The above ground biomass of MT droughted plants was reduced to 15% of the control, compared to 23% in WELL (Fig 7c-d). This difference was greater when comparing leaf area expansion; the leaf areas of plants subjected to soil drought reached 16% (MT) and 30% (WELL) of the well-watered treatment (Fig 8a). The value of average leaf area for MT largely reflects the contribution of the only plant that recovered from wilting.

$\Delta^{13}C$ in shoot dry matter was similar between genotypes under well-watered conditions, but reduced under drought, *i.e.* plants under drought discriminated less than control ones against the heavier ^{13}C isotope during C assimilation. While in MT there was a non significant decrease of 0.5 ‰, in WELL the drecrase was 1.8 ‰ (Fig 8b). WUE thus increased considerably more in WELL than in MT under drought conditions.

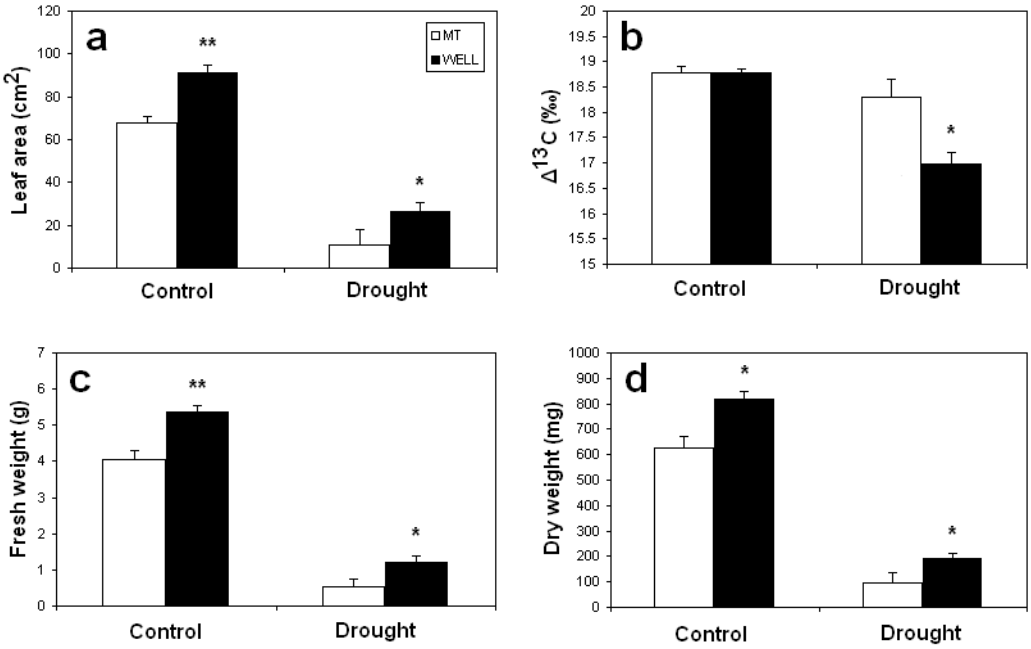

Figure 7. Experiment 5. Response to long-term drought in WELL compared to MT. (a) Leaf area. (b) Carbon isotope discrimination. (c) Aboveground fresh weight. (d) Aboveground dry weight, measured on day 42 after germination. * and ** indicate significant differences at p <0.05 and p <0.001 respectively calculated with Tukey's HSD test. Bars represent s.e.m. (n=5).

This experiment demonstrated that WELL leaves were better able to maintain growth and retain function under severe water stress, with delayed wilting and enhanced ability to recover from it. As plants of both genotypes initially had similar leaf area, these results further strengthen the case for enhanced stomatal closure in WELL under water stress, enabling leaves to maintain turgor for longer, and slow down the depletion of soil water. A final experiment (experiment 6) was designed to monitor stomatal conductance during soil drying in parallel with soil water potential.

3.3.6. The stomatal response to drought is increased in WELL

In this experiment plants were grown in a growth chamber, under constant irradiance, temperature and air humidity, and soil drought was imposed by withholding watering for seven days 42 and 49, while control plants were re-watered daily. Pre-dawn leaf water potential (Ψ_{pd}) was measured, as it is a surrogate for root water potential in the absence of transpiration and thus, ultimately a measure of soil water potential (Ψ_s) in the vicinity of the root (Boyer, 1995; Hinckley et al., 1978).

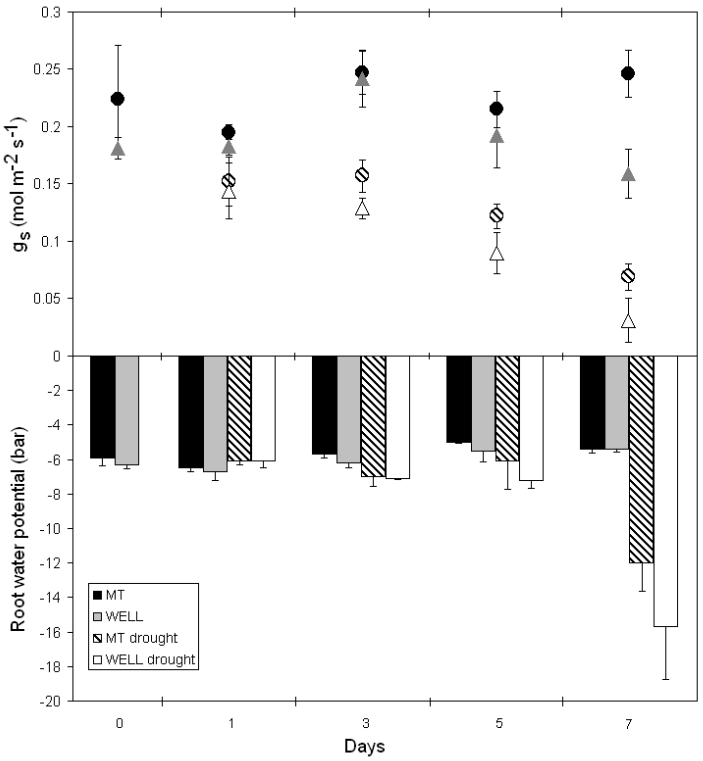

Figure 8. Experiment 6. Relationship between root water potential (measured as pre-dawn leaf water potential) and stomatal conductance to water vapour (g_s), in MT and WELL after the suspension of watering (day 0, 42 days after germination). Bars represent s.e.m. (n=5).

Under well-watered conditions, g_s was similar in MT and WELL, although with a trend for lower g_s, in WELL possibly due to differences in plant leaf area (MT 408 ± 23 cm²; WELL, 504 ± 43 cm²; p=0.083), or indicating a lower constitutive stomatal conductance in WELL. WELL showed a lower g_s at all 5 time points under well-watered conditions, in fact, the largest absolute difference in g_s between genotypes was observed for the well-watered treatment on day 7.. Upon suspension of watering, g_s decreased in both genotypes, but more rapidly in WELL (Fig 8), indicative of a faster stomatal closure, given that stomatal density was similar for MT and WELL (Fig 9). Sand volumetric water content measured at day 7 was not significantly different between genotypes, within treatments,. To remove a potential confounding effect of differences in plant leaf area,, leaves of the same rank and similar area were detached from the plants to compare their rates of water loss, in a controlled, constant environment.

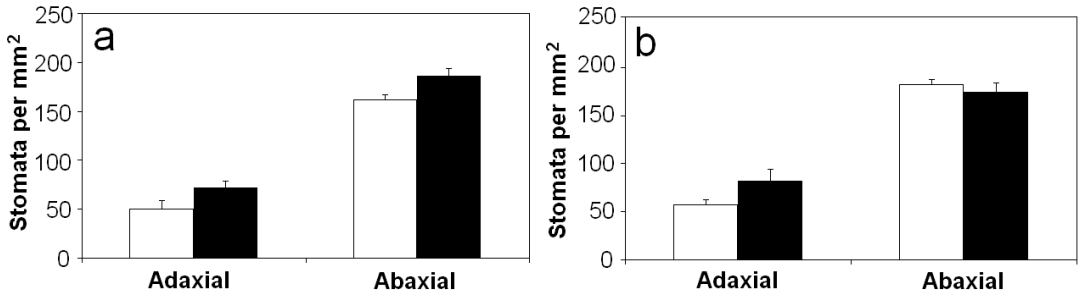

Figure 9. Experiment 6. Stomatal density for (a) well-watered and (b) drought in mature and fully-expanded leaves of MT (open bars) and WELL (closed bars). Differences not significant at p<0.05, calculated with Tukey's HSD test. Bars represent s.e.m. (n=4).

The rate of water loss from detached leaves of well-watered plants was measured over a period of 40 minutes after leaf detachment. The accumulated water loss per unit leaf area was lower for WELL than MT over this period (Fig 10).

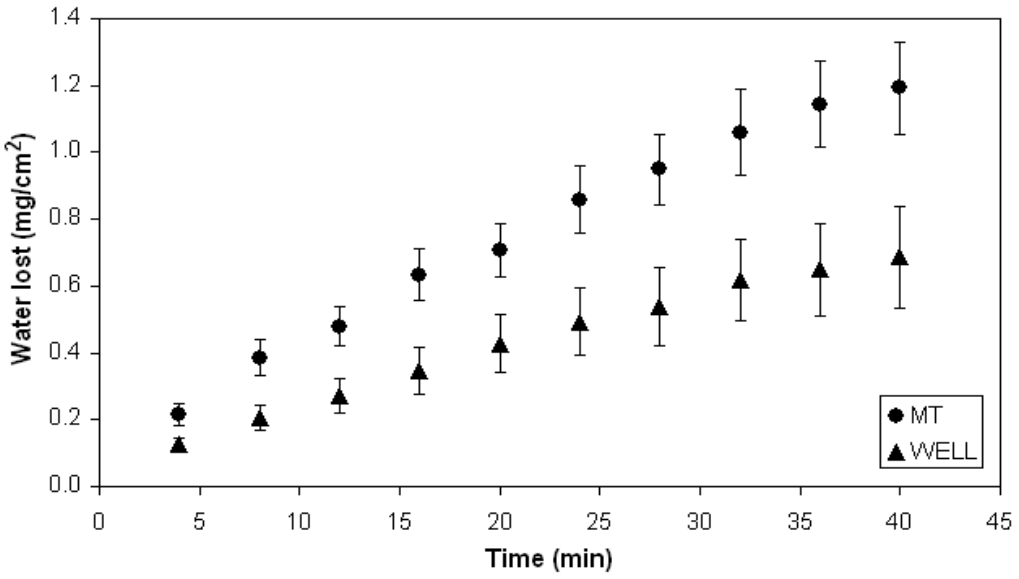

Figure 10. Experiment 6. Cumulative water loss from detached leaves of well-watered MT and WELL plants. Measurements conducted on fully-exposed terminal leaflets detached 49 days after germination. Bars represent s.e.m. (n=3).

3.4. Discussion

Growth parameters, leaf gas exchange and water-use efficiency (WUE) were measured in the new introgression line (WELL) and its parental genotype, Micro-Tom (MT) to determine the causes of delayed wilting shown by WELL leaves under drought. The first and most obvious parameter to be examined was total plant leaf area, which is a determinant of the transpirational power of the plant. (Nakazato et al., 2008)) compared days to

wilting in *S. lycopersicum* and *S. pimipinellifollium* and found that the species with the greatest leaf area, *S. lycopersicum*, wilted considerably earlier. In tomato, leaf area is greatly influenced by environmental conditions (Picken et al., 1986). Considerable variation was observed for this parameter between experiments. WELL had a reduced total leaf area before flowering, although this was not the case for all experiments (Table 2). This could, in principle and all other parameters being equal between genotypes, contribute to the delayed wilting of WELL compared to MT. The original observations of delayed wilting (Chapter 2) were done in full-grown plants after flowering, at a stage when total leaf area was either similar or significantly increased in WELL. In experiment 1 there was no difference in the average amount of transpired water per plant, before or after flowering. In spite of this, owing to a larger accumulation of dry mass, WUE was increased in WELL in the period between 30 and 50 days after germination. The proportion of leaves to stems decreases as the plant grows, so it is possible that in a semi-determinate genotype such as WELL, the increased stem elongation and production of additional sympodia drove the increase in WUE. Increased leaf dry weight also contributed, WELL leaves having a lower specific leaf area (SLA), *i.e.* lower area per unit dry weight. SLA has been found to be negatively correlated with WUE in savanna species (Hoffmann et al., 2005) and *Arachis* spp. (Wright et al., 1994). Likewise, increases in LMA (leaf mass area, the reciprocal of SLA) have recently been found associated with decreased cuticular water loss in Mediterranean tomato accessions (Galmés et al., 2011). Differences in the ratio of mesophyll

to stomatal conductance were found to play an important role in WUE in drought tolerant versus less tolerant Mediterranean tomato accessions (Galmés et al., 2011). In this work possible differences in cuticular losses and mesophyll conductance in relation to different leaf anatomies and trichome types, density and distribution were ignored. this is an area of interest for further investigation in the future. Under drought conditions, low SLA is usually the result of a thickening of the cuticle and the epidermis, or both; increased fraction of sclerenchymatic tissue; and more tightly packed mesophyll cells with thicker cell walls (Niinemets and Sack, 2006). Studying 15 near-isogenic lines of *Solanum habrochaites* introgressed in tomato, (Muir and Moyle, 2009) Muir and Moyle (2009) concluded that in spite of a significant positive correlation between decreased SLA and delayed wilting, there was no mechanistic association between both traits and that the relationship should be attributed to other unknown linking variables. No significant difference was observed in leaf thickness between MT and WELL, but WELL appeared to have longer palisade mesophyll cells. Contradictory reports exist for SLA in *S. pennellii* (Comstock et al., 2005; Kebede et al., 1994; Torrecillas et al., 1995) and it is likely that this trait is heavily influenced by the environment (Niinemets and Sack, 2006).

Stomatal conductance (g_s) is a component of transpiration efficiency (*TE*) through its control of rate of water loss and limitation to CO_2 uptake (Cowan and Troughton, 1971; Farquhar and Sharkey, 1982). A considerable decrease in g_s was observed upon fruit set in well-watered glasshouse-grown

WELL plants compared to MT (Experiment 1). *TE* was thus increased in WELL, although there was a concomitant stomatal limitation to photosynthesis, which explains the different magnitudes of the two effects (g_s was reduced 44% but *TE* increased only 13%). A second assessment of g_s, this time in plants of both genoytpes grown in a single pot, showed a lower g_s for WELL leaves (Experiment 3). Two further sets of gas exchange measurements were obtained under the more tightly controlled environmental conditions of a growth chamber,m and in both cases a lower g_s was observed in WELL, although this time the difference was only statistically significant for plants under drought (Experiments 4 and 6). This discrepancy between growth-chamber and glasshouse-grown plants (Experiments 1 and 3) warrants a careful interpretation. There are some indications that the glasshouse plants may have inadvertently suffered from transient water stress. In the glasshouse experiment (Experiment 1) measurements were performed on two-month old plants which were already beginning to set fruit. Leaf senescence was also evident in the bottom leaves. It is well-known that hydraulic conductance, especially in the roots, decreases as tomato plants age (Rudich et al., 1981). Plants grown in sand and watered once a day have also been reported to develop roots of limited size and hydraulic conductivity (Bar Yosef et al., 1980). Further, the low relative humidity in the glasshouse throughout the experiment (20% and 40%, average for day and night, respectively) probably resulted in a high evaporative demand which can effect stomatal closure through a 'feedforward' mechanism (Farquhar, 1978; Lange et al., 1971). Interestingly, *S.*

pennellii leaves have been shown to have a strong response to variations in air humidity (Rick, 1973).

An effect of plant architecture in water relations late in the growth cycle could not be ruled out either. To test whether growth habit leads to a difference in WUE between genotypes, a nearly-isogenic indeterminate MT line was compared to the determinate MT. Under well-watered conditions in the glasshouse, no difference was observed in long-term WUE. Determinate MT and indeterminate MT sp^{+}/sp^{+} plants were harvested at a point when both genotypes had a similar leaf area, although distributed over a different number of leaves. It is contentious whether the *SP* gene affects axillary branching (Lifschitz, 2008; Périlleux, 2008). The results shown in this chapter suggest that axillary branching is reduced in plants with a functional *SP* gene compared to *sp*/*sp* plants. This reduction concurs with the observed reduction of *SP* expression in axillary meristems once they start to grow (Thouet et al., 2008). This, of course, does not rule out the possibility that a semi-determinate plant could have an increased WUE compared to determinate and indeterminate ones. The comparison between MT and MT sp^{+}/sp^{+} does not answer the question. The results only show that determinacy does not *per se* influence leaf water relations under the experimental conditions of this work. It is not a thoroughly explored issue whether growth habit influences long-term WUE in crop plants, although it is of interest, since many crops, not only horticultural, have been modified through domestication to grow in determinate fashion (Doebley et al., 2006).

Indeterminate growth is typical of natural environments and it could provide plants with flexibility to deal with fluctuating and unpredictable conditions (Stebbins, 1974).

Stomatal conductance responds to a combination of hydraulic and chemical signals conveyed from the roots (reviewed in Tardieu and Davies, 1993). Water content in the soil does not necessarily describe the availability of water for the plants, nor how the water moves within the soil profile (Kirkham, 2005). A better parameter is soil water potential (Ψ_s) in the rooting zone, which can be estimated by pre-dawn leaf water potential (Ψ_{pd}). The assumption is that the water potential gradients in the plant disappear during the night when transpiration stops, and thus Ψ_{pd} is equivalent to Ψ_s (Boyer, 1995; Hinckley et al., 1978). A curve can be drawn (Fig 8) relating the response of g_s to decreasing Ψ_s, after the classic drying curves of Slatyer (Slatyer, 1967) as done for Experiment 6. In that experiment there was an apparent lag in the reduction of Ψ_s after suspending watering, although g_s decreased immediately on the first day. This is probably because the measurements of water potential (early morning) preceded those of g_s (mid-afternoon). The use of sand in the pots should also be discussed. Sand will usually bind water mainly by capillarity and therefore release most of the water at relatively higher water potentials. A sand water retention curve (van

Genuchten, 1980) shows that the difference between 'field capacity', when the soil is saturated with water after draining, and 'permanent wilting point' when no more water is available for the plant, is very small. Thus, the water-holding capacity of a sandy soil is low and suspending irrigation will induce water deficit in plants more suddenly than in other soil textures. Ψ_s was never significantly different between genotypes in the drought treatment (Fig 8), suggesting that both depleted the soil water at a similar rate. In spite of this, g_s was always lower in WELL than in MT. Under a longer drying cycle, in a soil with higher water retention, g_s in MT will likely fall to a very low level like that of WELL, but the period during the drying cycle when WELL has lower g_s, will differ in duration depending on potential transpiration rate versus available water stored in the pot, and may depend on speed of onset of stress, *i.e.* water release curve of the pot medium. There is also growing evidence against the main assumption behind the determination of Ψ_s via Ψ_{pd}, namely absence of night-time transpiration (Donovan et al., 2003). Its occurrence has been shown in various species (Daley and Phillips, 2006), including tomato and its relatives, where, interestingly, the drought-adapted species *S. pennellii* and *S. sitiens* had the highest rates of night-time water loss (Caird et al., 2007).

Rates of water loss from detached leaf determinations were compared to assess the performance of each genotype at the leaf level, where the whole

plant architecture cannot affect water relations. Plots of leaf drying are usually biphasic, consisting of an initial rate of water loss before stomata react to leaflet dehydration, then a second phase where stomata have shut and water loss is due to cuticular transpiration. This was not observed in this experiment, probably because the measurement was performed for only 40 minutes, while usually to see bi-phasic curves one needs measurements over 3 or 4 hours, which is the conventional length of time such measurements cover to reach asymptotic values (Fig 10). WELL leaves lost water more slowly than MT. As WELL consistently showed similar or higher stomatal densities than MT (Appendix 3C) these data suggest its stomata either respond faster or close more than those of MT. This is consistent with the delayed wiltingearly during the introgression process (Chapter 2, Fig 1). Kahn et al. (1993) also showed that in detached leaves that were wilted to 88% of their fully-turgid weight, *S. pennellii* maintained a higher leaf water potential and accumulated less ABA than *S. lycopersicum* or hybrids of the two species. Such water conservation mechanism may have been introgressed into MT. The drawback of detached leaf analyses is that conditions are quite different to those of the leaf *in planta* so it is hazardous to draw too many conclusions from such experiments. Another approach was taken in Experiment 5 where, to avoid confounding effects of plant size on water relations when comparing the two genotypes, water deprivation was imposed at the stage of two leaves. Furthermore, plants were grown in a soil mix with high water retention capacity and under mild evaporative demand in a glasshouse, so that water stress set-in more slowly and built up over a

longer period of time than in previous experiments (weeks instead of days). After a drought period with two intervening periods of watering, it was observed that: a) most WELL plants reached a more advanced stage of development (flowering) than MT; b) the reduction in leaf area and aboveground dry mass was 50% less in WELL than MT (Fig 8a), and c) $\Delta^{13}C$ in total above ground dry matter was more reduced in WELL than MT (Fig 8b). These results show that the *S. pennellii* introgression in WELL improves plant survival and growth under drought, even before flowering, when there is no difference in growth habit between the two genotypes. This suggests a higher sensitivity of stomata to reduced water supply in WELL, which leads to a greater decrease in g_s than in MT. One way in which this could happen is increased ABA levels or sensitivity to ABA in guard cells, or another physiological alteration which could indirectly result in more rapid redistribution of ABA to the guard cells (*e.g.* pH changes, reviewed in Hartung et al. 1998). ABA-overproducing tomatoes have been produced by driving the transcription of the ABA biosynthesis gene *LeNCED1* with a Gelvin Superpromoter (Thompson et al., 2000). Their most remarkable feature is a decrease in g_s which results in increased *TE* and drought resistance (Thompson et al., 2007). There are phenotypic similarities between WELL and the *LeNCED1* transgenic plants, such as more erect leaves and increased leaf dry weight, but also some important differences, such as considerably longer petioles and reduced epinasty in the transgenic that were not oberved in WELL., Increased ABA levels also result in lower g_s in the *LeNCED1*

transgenic plants even under well-watered conditions (Thompson et al., 2007)ı, so the question of possible differences in ABA synthesis and/or signalling between WELL and MT warrants further investigation No ABA hypersensitive mutants are known in tomato, but several have been described in Arabidopsis (Finkelstein and Rock, 2002). A large number among them are a by-product of alterations in genes for other hormonal pathways, especially ethylene, which has strong cross-talk with ABA (Ghassemian et al., 2000). Among the *bona fide* specific ABA mutants, *abh1* carries a mutation in a gene for a nuclear mRNA cap binding protein that modulates the transcription of early ABA signalling elements (Hugouvieux et al., 2001). The *abh1* mutant is not highly pleiotropic, showing only weakly serrated leaves and a slightly slower growth than wild type plants, but considerably reduced g_s and wilting under drought stress (Hugouvieux et al., 2001).

The drought tolerance and higher WUE of *S. pennellii* have been ascribed mostly to changes in the anatomy and morphology of its leaves (Kebede et al., 1994). The leaves of WELL do not look different from those of MT at first glance, but there are subtle structural differences. Tomato normally has a high stomata and trichome density (about two thirds of the total) on the abaxial or lower epidermis and a lower density (one third) on the adaxial or upper epidermis of the leaf, whereas *S. pennellii* has a balanced distribution between both sides (Gay and Hurd, 1975; Kebede et al., 1994). WELL leaves showed a minor, yet statistically significant, increase in trichome density on the adaxial surface of the blade. This effect was

consistent across different experiments, under different conditions (Appendix 3C). The stomatal density was strongly dependent on growth conditions, and it (Gay and Hurd, 1975)tended to be higher in WELL than in MT, especially for the adaxial epidermis, except in Experiment 1 where the increase was observed on the abaxial and not the adaxial side. As for a role of the internal features of the leaf, the palisade cell density was not quantified, but in WELL the cells are taller and appear to be more tightly packed (Appendix 3B).

As mentioned in Chapter 2, several other factors could also contribute to the delayed wilting in WELL, such as soil exploration by roots, hydraulic resistances and osmotic adjustment. These were not considered here but should be the subject of further experiments.

3.5. Conclusion

The data presented in this chapter show that WELL has a higher WUE than MT under conditions of limited water supply. WELL tends to have lower g_s when there is no water limitation, and under water stress the difference becomes more marked and leads to slower soil drying, delayed wilting and prolonged maintenance of leaf growth. This happens at late or early stages of development and in spite of WELL having a similar or higher stomatal density than MT. Thus, the lower stomatal conductance of WELL leaves does not appear to be determined by anatomical or morphological

features of the leaves, although minor differences were observed relative to MT leaves.

The genetic basis of the phenotypes described in this and the earlier chapter will be explored in the next chapter, and the WELL introgression will be mapped on the tomato genome and broken into smaller *S. pennellii* segments through recombination to narrow down a candidate region for the phenotype of interest in WELL, *i.e.* reduced wilting and higher WUE, and to examine whether the different phenotypes associated with WELL are genetically linked and caused by a single gene, or arise from allelic variation at several linked genes. Differences in stomatal responses to water deficit between WELL and MT may involve gene(s) that also affect determinacy, but have an effect at the molecular level before the growth habit difference is macroscopically evident.

Chapter 4 – Mapping and genetic analysis of WELL

4.1. Introduction

WELL has several distinct phenotypes: developmental (height, semi-determinacy, leaf angle, fruit colour) and physiological (water relations and stomatal control), which could be due to a single gene with pleiotropic effects or of several genes introgressed from *S. pennellii*. The results presented in Chapter 3 showed that delayed wilting of WELL leaves was caused by an increased stomatal sensitivity to water deficit, leading to reduced transpiration rates and water conservation. WELL leaves have an enhanced ability to maintain turgor under a given soil water potential even in young seedlings of similar leaf area, suggesting that the delayed wilting and morphological phenotypes of WELL may be under control of different, but closely linked genes. In this chapter, a genetic approach was used to map the introgression and find recombinants with smaller introgression sizes where the multiple phenotypic effects of WELL are separated.

The genetic structure of the tomato plant allows detection of a large array of hereditary variations. Since tomato is a diploid species, monogenic mutations of many types can be readily distinguished phenotypically (Stevens and Rick, 1986). A collection of 1023 monogenic mutants is maintained by the C. M. Rick Tomato Genetics Resource Centre at the University of California, Davis (http://tgrc.ucdavis.edu/). The first genetic map of tomato showing the relative positions of 153 morphological and physiological markers on all 12 chromosomes was published in 1968 (Butler, 1968). A second generation of

markers, isozymes, became popular during the 1970s, but had only a limited application due to the low number of markers available (36) and the low level of polymorphism between closely-related genotypes (Stevens and Rick, 1986; Tanksley and Orton, 1983). Mapping resolution was considerably increased with the advent of DNA marker technology in the 1980s, and as of 2011 the molecular linkage map of tomato contains 2,500 mapped molecular markers (http://solgenomics.net).

Over the last decade, the development and use of PCR-based markers has become routine in tomato, as these are cheaper, faster and less labour-intensive in their use than previous DNA markers such as RFLPs (Restriction Fragment Length Polymorphisms). The availability of the fully sequenced genome of Arabidopsis (*Arabidopsis thaliana*) has allowed the identification of a set of more than 1000 genes (Conserved Ortholog Set, COS) in single or low copy number from Arabidopsis (*Arabidopsis thaliana*) with a conserved sequence in tomato (Fulton et al., 2002)have been identified . A large number of COS markers have been mapped using an F_2 population of tomato (*Solanum lycopersicum*) × *S. pennellii* and are available online through the Sol Genomics Network (http://solgenomics.net). Polymorphism between tomato and *S. pennellii* for these markers can be revealed by analyzing PCR products for cleaved amplified polymorphic sequence (CAPS) markers or sequence characterised amplified region (SCAR) markers (Frary et al., 2005). CAPS markers are scored based on variation in the size of fragments following digestion of the PCR products by a restriction endonuclease

(Konieczny and Ausubel, 1993). CAPS markers can also be derived from DNA sequences of mapped RFLP markers, thus eliminating the time-consuming DNA blotting step (Frary et al., 2005; Komori and Nitta, 2005). SCAR markers are usually based on amplicon size at a given genetic locus (Paran and Michelmore, 1993). Differences in PCR product length are revealed using standard agarose gel electrophoresis. Both types of markers are usually co-dominant and can thus be used to distinguish between plants that are homozygous or heterozygous for the marker alleles. SCAR analysis is relatively inexpensive and straightforward compared with CAPS, because treatment with restriction enzymes is unnecessary after PCR (Agarwal et al., 2008). Lastly, the sequence of the tomato genome is now publicly available, making design of markers in known map positions more straightforward (http://solgenomics.net).

The aims of this chapter were to map WELL to one of the 12 tomato chromosomes; to determine the size of the *S. pennellii* introgression; to generate recombinant lines with a reduced introgression size; and to conduct an initial characterisation of the recombinant lines, especially with respect to transpiration rates and morphology.

4.2. Methods

4.2.1. Plant material

Solanum lycopersicum L. cv. Micro-Tom (MT) and the WELL introgression line, generated as described in Chapter 2, were used for the experiments. The ABA-deficient mutant *sitiens* (introgressed into MT from its original background, the cultivar Rheinlands Rhum) was kindly provided by Dr. Lázaro Peres (University of São Paulo, Brazil).

4.2.2. Growth conditions

Plants for crosses or for DNA extraction were grown in a glasshouse with 25°C/20°C (day/night) temperature, 11.5 h photoperiod and 250 to 350 μmol photons m^{-2} sec^{-1} PAR irradiance. Ambient relative humidity fluctuated between 40 and 60%. Seeds were surface-sterilized by treatment with a 5% (v/v) solution of household bleach (White King, Australia) for 5 minutes and then washed in distilled water. Seeds were sown in 40×20×5 cm trays filled with seed raising mix (Debco, Victoria, Australia). After the appearance of the first pair of true leaves, seedlings were transplanted to 350 -ml pots containing a 1:1 soil:vermiculite mix supplemented with 1 g L^{-1} 10:10:10 NPK fertilizer and 4 g L^{-1} lime.

4.2.3. Phenotyping strategy for mapping WELL and the physiological evaluation of the recombinants

Three experiments were conducted with the aim of associating chromosome regions derived from *S. pennellii* with physiological traits in recombinant lines of WELL. Growth conditions for each experiment are detailed in Appendix 4A.

In Experiment 1, a segregating F_2 population derived from the cross MT × WELL was grown alongside MT and WELL controls, and watered by capillarity. Genomic DNA was extracted from each plant. After flowering, plants were scored for height and classified as tall, intermediate or short, using the MT and WELL control plants as standards (MT=11 cm; WELL=18 cm; intermediate ~14-15 cm). Watering was withheld at the beginning of fruit set (75 days after seed germination) to assess wilting at a similar stage as in the initial screen leading to the identification of WELL (see Chapter 2). Wilting was scored as explained in Results section 4.3.2.2. After analysing the height and wilting data, the most promising individuals were genotyped as explained below (Section 4.2.5). Fruits were harvested individually for each plant and seeds cleaned and stored. Identified recombinants were selfed to isolate homozygous lines, whose seeds were then used for initial phenotyping (Experiments 2 and 3).

Experiment 2 was done to analyse the response to soil water deficit of a recombinant line with a reduced *S. pennellii* introgression (line #54). Recombinants and MT control plants were grown in individual pots placed in trays and watered by capillarity. At the stage of five leaves (22 days after

germination), total projected leaf area for each plant was estimated as the product of length × maximum width of each leaflet (after verification that the relationship between that product and the actual area was conserved across lines). The plants of each genotype (MT and #54) were then split in two batches (control and drought) of seven individuals each, chosen so that all batches would have the same average leaf area. Plants allocated to the drought treatment were not watered for seven days, whereas the control plants were kept well-watered, by capillarity. The progression of wilting was assessed visually and at the end of the drought period all the plants were re-watered for leaves to regain sufficient turgor for reliable measurements of leaf area. Plants were harvested and dried, and carbon isotope discrimination was determined (described in Chapter 3) for leaves developed entirely during the water withdrawal period.

The aim of Experiment 3 was to study the response to drought in six more homozygous recombinant lines with reduced *S. pennellii* introgressions, for which seeds became available later than for line #54 above. MT was used as a control. Unlike the previous two experiments which were conducted in a glasshouse, this experiment was performed under more controlled conditions in a growth chamber (Appendix 4A). Seedlings of MT and the six recombinant lines (#4, #43, #45, #48, #63 and #130) were grown in individual pots and watered by sub-irrigation from a tray. At the four leaf stage (20 days after germination), leaf area was estimated as in Experiment 2, plants of each genotype were divided in two batches and water

was withheld for one of them as described above. The soil surface was covered with a plastic film and pots were weighed daily. Water loss through transpiration was calculated after subtracting water lost by control pots without plants. Seven days after the start of the suspension of watering, plants were rewatered, leaf area was again measured and aboveground organs were oven-dried for 48h. The rate of leaf expansion over the 7-day period was calculated, and with the values for leaf area, the rate of daily water loss per unit leaf area (transpiration) was estimated. Specific leaf area (SLA), the ratio of leaf area per unit leaf dry mass was determined for individual leaves expanded before and after the beginning of the drought treatment.

4.2.4. Mapping the *S. pennellii* introgression in WELL

When the chromosome *1* location of WELL was confirmed (see section….below), the span of the introgressed segment was estimated by genotyping WELL, MT and *S. pennellii* with a set of COS markers (Fulton et al., 2002; Appendix 4C). The same markers were used to screen individuals of an F_2 population derived from the cross MT×WELL to identify recombinant sub-lines.

Total genomic DNA was extracted from young leaflets following the protocol of Edwards et al. (1991). PCR amplification was conducted on a MJ

Research PTC-200 thermal cycler using in 25-µL volume reactions, including 1 µL (up to 200 ng) of template DNA, 5 µL 5× reaction buffer (GoTaq Flexi Green, Promega), 0.5 µL 25 mM $MgCl_2$, 0.5 µL 10mM dNTPs (Promega), 0.5 µL of each forward and reverse primer (see Appendix 4C for primer sequences) from 10 µM stocks (Invitrogen), 16.8 µL MilliQ H_2O and 0.1 µL (5 units/µL) DNA polymerase (GoTaq, Promega). The cycling conditions were 94 °C, 2 min; 35× (94 °C, 30s; 55 °C, 1 min; 72 °C, 2 min); 72 °C, 10 min. When required by the protocol (CAPS, Appendix 4C), 1 µl of PCR product was used for enzymatic digestion, which was performed in 20 µL reactions following the manufacturer's recommendations (NEB, Bethesda, USA). PCR products were visualized in 1% agarose (Invitrogen) gels run at 80 mV for 1h and stained with ethidium bromide.

4.2.5. Statistical analyses

Student's *t*-test and all other statistical analyses (outlier tests, probability distribution) were performed with the online software GraphPad (http://www.graphpad.com/quickcalcs/index.cfm). The principal component analysis was performed using the NumPy package for Python 2.7.1 following the guidelines of Abdi and Williams (2010). The data was 'centred' (*i.e.* mean values were subtracted from the variable columns), the

whole matrix was normalised to the square root of the number of genotypes, and each variable column was normalised – for further details about the procedure see Abdi and Williams (2010).

4.3. Results

4.3.1. Mapping of WELL

4.3.1.1. The WELL introgression maps in the vicinity of the *y* gene on chromosome *1*

Early during the introgression process it was apparent that ripe fruits were of a consistently lighter colour in WELL than MT (Chapter 3). Peeling and visually inspecting the fruit revealed a colourless epidermis - typical of a mutation in the yellow fruit epidermis pigmentation gene (*y*) present in *S. pennellii* - as opposed to the yellow pigmentation conferred by the wild-type gene (y^+) in the fruit epidermis of most cultivated tomato varieties, including MT (Rick and Butler, 1956). The *y* mutation is a classic morphological marker of tomato (Rick and Butler 1956) and a strong candidate gene has been recently identified and mapped to an interval of 28.5 cM on the short arm of chromosome *1* (Ballester et al. 2010)(Rick and Butler, 1956). This suggested that the introgression from *S. pennellii* could be located on chromosome *1*.

WELL was thus test-crossed with a chromosome *1* marker stock carrying the recessive *sitiens* (*sit*) mutation which had previously been introgressed into MT from its original background in cv. Rheinlands Rhum (Peres et al, unpublished). The normal function of SITIENS is to catalyze the oxidation of ABA-aldehyde into ABA; thus, homozygous mutant plants have a wilty phenotype even under well-watered conditions, as stomata have lost the ability to close (Taylor et al., 1988). The mutation is recessive and was mapped to the short arm of chromosome *1* (Balint-Kurti et al., 1995; Tanksley et al., 1992) and a candidate gene for *sit* has recently been identified (Adato et al. 2009). The segregation ratio of the F_2 progeny of a cross between homozygous *WELL, y* and *sit* parents (*WELL*$^+$/*WELL*$^+$ *y*/*y* *sit*$^+$/*sit*$^+$ × *WELL*/*WELL* *y*$^+$/*y*$^+$ *sit*/*sit*) was analysed to test the chromosomal location of WELL and determine its relative position with respect to two known morphological markers, *sit* and *y*, on chromosome *1*. Plants were visually scored as *WELL* when they showed the tall, semi-determinate phenotype and *WELLl*$^+$ when they had the MT phenotype, *i.e.* short and determinate (Fig 1). 180 F_2 plants were grown and scored for three phenotypes (Fig 1): *WELL* (tall/short), *y* (red/pink fruit), *sit* (turgid/wilty). Linkage was assessed by observation of phenotypic ratios and a χ^2 test (Table 1).

Figure 1. Representative F_2 plants derived from the cross *WELL*$^+$/*WELL*$^+$ *y*/*y* *sit*$^+$/*sit*$^+$ × *WELL*/*WELL* *y*$^+$/*y*$^+$ *sit*/*sit* The phenotypes of *WELL* and *sitiens* are shown, *y* was scored in the fruit. (a) *WELL*$^+$ *sit* (a Micro-Tom plant homozygous for the *sitiens* mutation); (b) *WELL sit* (*WELL* plant homozygous for the *sitiens* mutation); (c) *WELL*$^+$ *sit*$^+$ (*i.e.* Micro-Tom) and (d) *WELL SIT*$^+$ (*i.e.* *WELL*).

Table 1. Segregation of WELL and morphological markers *sitiens* and *yellow* in an F_2 population from the cross P1 (*WELL* $^+$/*WELL*$^+$ *y* /*y sit*$^+$/*sit*$^+$) × P2 (*WELL*/*WELL y*$^+$/*y*$^+$ *sit* /*sit*).

p=0.05 χ^2=3.84 with 1 d.f. for 3:1 segregation (bottom row) and for independent assortment between pairs of genes (far right column). * indicates non-independent assortment.

Phenotype	*WELL*$^+$	*WELL*	*sit*$^+$	*sit*	*y*$^+$	*y*	χ^2 i.a.
y$^+$	101	39					14.22 *
y	40	0					
WELL$^+$			112	29			0.52
WELL			33	6			
sit$^+$					116	29	2.13
sit					24	11	
Total	141	39	145	35	140	40	
χ^2 3:1	1.06		2.96		0.74		

The chi-square results for Mendelian segregation show that, as expected, both marker genes (*y* and *sit*) segregated in a 3:1 ratio. *WELL* also segregated in a Mendelian 3:1 fashion, suggesting that, at least for its plant height component, *WELL* is controlled by a single dominant gene (Table 1). A significant deviation from independent assortment was observed for the

combined segregation ratio of *WELL* and *y* (χ^2=14.22), confirming the chromosome *1* location of *WELL* in the vicinity of the *y* locus (Table 1). The position of *WELL* relative to the *sit* and *y* loci was then estimated by calculating the recombination frequency between each pair of genes (Table 2, details in Appendix 4B).

Table 2. Calculation of recombination frequency (r) between each pair of genes. See Appendix 4B for details of calculations.

Gene pair	*WELL - y*	*WELL - sit*	*sit - y*
Phase	Repulsion	Coupling	Repulsion
Expected Frequency	$(1 + r^2)/2$	$[1 + (1 - r)^2]/2$	$(1 + r^2)/2$
Observed Frequency	(101+0)/180=0.561	(112+6)/180=0.655	(116+11)/180=0.705
r	0.349	0.443	0.641

The r values allow for the relative positioning of *WELL*, *sit* and *y* along the chromosome (Sturtevant, 1913). These positions are shown as a linkage map in Figure 2.

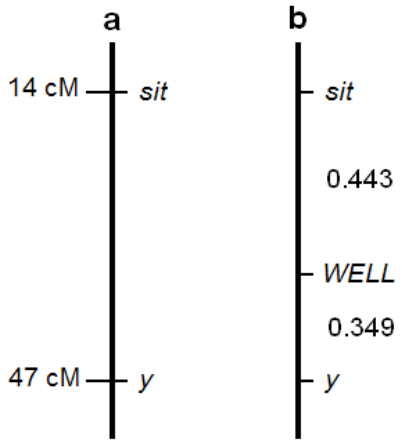

Figure 2. Genetic maps depicting (a) positions of morphological markers *sit* and *y* on a classical map of chromosome *1* (Balint-Kurti et al., 1995), and (b) estimated position of *WELL* calculated by linkage analysis with *sit* and *y*. One centimorgan (cM) is defined as the genetic distance between two loci with a statistically corrected recombination frequency (r) of 1%; the genetic distance in cM is numerically equal to r expressed as a percentage. NB: at genetic distances greater than about 7 cM, the relationship between r and genetic distance is no longer linear.

4.3.1.2. The introgression from *S. pennellii* in WELL encompasses 42-54 cM on chromosome *1*

Once the chromosome 1 location of the WELL introgression was confirmed, it was necessary to estimate its length and position, so WELL, MT and *S. pennellii* were genotyped with the set of COS markers listed in Appendix 4C (Frary et al., 2005).

The results are shown in Figure 3 and are consistent with the data from the classical mapping (Fig 2). They show an introgression of between 42.5 and 54 cM including the centromere of chromosome *1* (Chang et al., 2007). The 7.5 cM and 4 cM regions of unknown genotype (grey segments) flanking the *S. pennellii* segment could not be narrowed down due to time constraints.

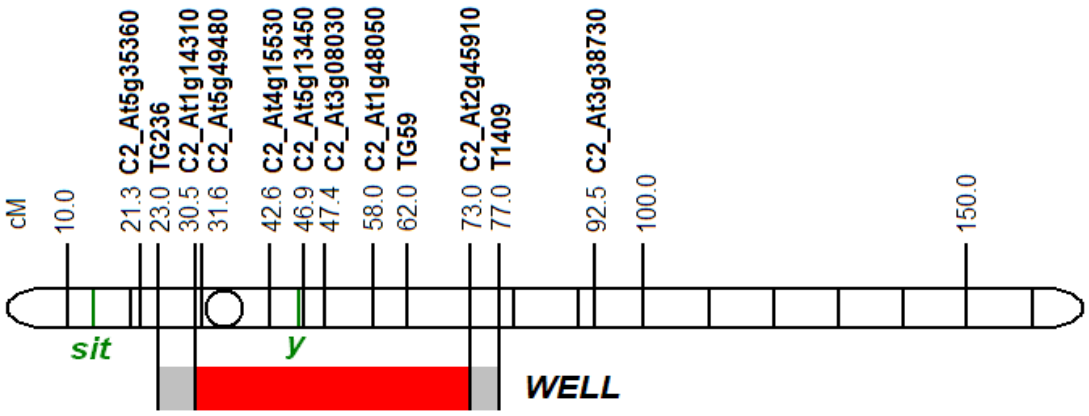

Figure 3. Schematic representation of tomato chromosome *1* showing the relative positions of 12 molecular markers used to map the introgression from *S. pennellii* in WELL (red bar). Numbers next to the marker names indicate the genetic distance in cM from the top of the chromosome according to the *S. pennellii* F_2 2000 map (www.sgn.cornell.edu). The position of the morphological markers *sit* and *y* is indicated in green. Grey bars represent flanking regions of undetermined genotype. The centromere (circle) was positioned after Chang et al. 2007. *sit* and *y* were positioned after Balint-Kurti et al. 1995.

4.3.2. Some traits associated with WELL can be separated through recombination

Given the large size of the *S. pennellii* introgression in WELL, the next step was to create a set of lines with smaller, overlapping introgressions to examine genetic linkage between the several distinctive phenotypes associated with the original introgression (Chapters 2 and 3). A first approach was to take advantage of the morphological marker *y* now known to be linked to *WELL* (Table 2) and use it to screen for recombinants visually.

4.3.2.1. Increased height maps to a small region in the WELL introgression close to the chromosome *1* centromere

A series of crosses between the original WELL line and MT was performed (Appendix 4D). After backcrossing WELL to MT, seeds from F_2 plants were harvested individually and a progeny test performed to find tall, red-fruited plants carrying *WELL* and *y* in coupling phase. A plant was found with very low fertility, which upon selfing produced only 18 seeds. Of these, only 11 seeds germinated and these plants were all tall but segregated for red versus pink fruit. It was deduced that these plants were homozygous for height and that some of them should also be homozygous for *y*. DNA was extracted from all 11 plants and the marker C2_At5g13450 was used to genotype them (Fig 4). This marker was chosen because of its position (46.9 cM) near the centre of the introgressed *S. pennellii* fragment (Fig 3) and also because of its convenience as a SCAR marker. One homozygous plant and three heterozygous plants for the *S. pennellii* allele of C2_At5g13450 were found (Fig 4, lanes 9; 4, 6 and 12 respectively). Seeds of the homozygous line (labelled #18 in the original batch of seeds) were sown and, as expected, all 15 plants were tall (MT, 14.86 ± 0.45 cm, n=7; #18, 20.83 ± 0.48 cm, n=10) and bore red fruit.

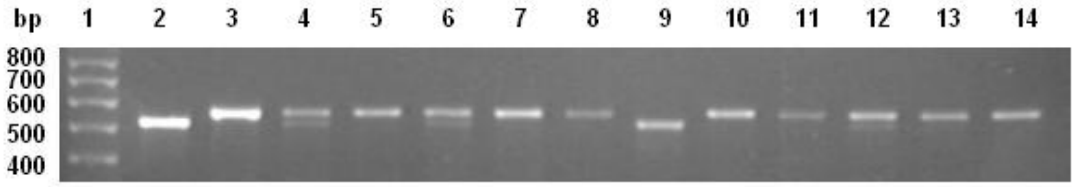

Figure 4. Genotypic screening of recombinant WELL plants. Marker C2_At5g13450, *S. esculentum* 500 pb; *S. pennellii* 550 bp. Lane 1: DNA 1 kb ladder; lane 2: MT; lane 3: *S. pennellii*; lanes 4-14: F_3 plants from the cross MT x WELL.

Although simple and effective, this approach, by itself, would not be enough to generate recombinants covering the whole original introgression with overlapping segments. Thus, a different strategy was employed: a segregating F_2 population derived from the cross MT×WELL was sown, characterised phenotypically (Section 4.3.2.2) and genotyped using chromosome *1* molecular markers (Section 4.3.3). The population consisting of 164 individuals was sown and grown as described in the Methods section (4.2.2). After losses due to early seedling mortality and disease (red spider mite and mildew) a total of 132 healthy plants reached maturity and yielded seeds. MT and WELL plants were grown alongside as controls.

4.3.2.2. Delayed wilting and height can be separated in a segregating F_2 population

Considerable phenotypic variation was observed among the F_2 plants. Segregation occured for traits such as plant height, number of leaves and fruits and total fruit fresh weight and shiny/glossy as opposed to matte leaves. Probably the most outstanding trait of WELL was its increased height

due to longer internodes. A drought experiment (Experiment 1) was performed on this population to determine whether there was a correlation between delayed wilting and increased height. Sixty-two days after seed germination, when plants were flowering, water was removed from all trays and watering was withheld for five days, after which wilting was assessed visually and by touch on a 0 to 3 scale (Table 3) adapted from Engelbrecht et al. (2007). The values were determined by two independent observers and then averaged, which resulted in intermediate categories (0.5, 1.5, 2.5).

Table 3. Experiment 1. Description of the categories used for the visual assessment of wilting. The values were assigned by two independent observers and then averaged, so some plants show intermediate values between those described in this table (*i.e.* 0.5, 1.5 and 2.5). See Figure 7a for examples of categories 0 and 3.

Category	Wilting stage	Visual characteristics
0	Turgid (not wilted)	No signs of wilting or drought stress
1	Slightly wilted	Slight leaf angle changes
2	Wilted	Strong leaf angle changes
3	Severly wilted	Very strong leaf angle change

Control MT plants were more wilted than WELL plants after five days without watering (Fig 5a). There was a considerable spread in the data for wilting in MT, yet the difference from WELL was significant at p=0.001.

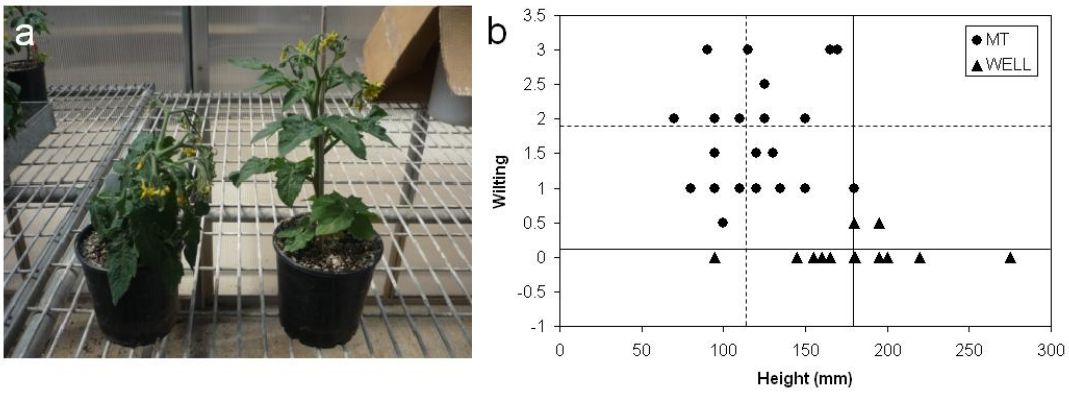

Figure 5. Experiment 1. Visual assessment of wilting after five days of withholding watering in MT and WELL plants. (a) Representative MT (left) and WELL (right) plants at the end of the drought period. MT was scored as '3' (severely wilted) and WELL as '0' (turgid). (b) Scatter plot showing the relationship between height and wilting in MT (n=21) and WELL (n=12). Each dot represents an individual plant. The lines represent the arithmetic mean for each parameter (MT, dotted line; WELL, solid line).

A normal distribution was observed for height (Fig 6b) in the F_2 population, although with two peaks close to the mean heights of MT (75-100 mm interval) and WELL (175-200 mm interval). These contradictory data are inconclusive and insufficient to warrant a conclusion on whether segregation of height is Mendelian or not. A smaller proportion of plants showed more severe wilting in this population (4/132) than the MT control population (4/21).

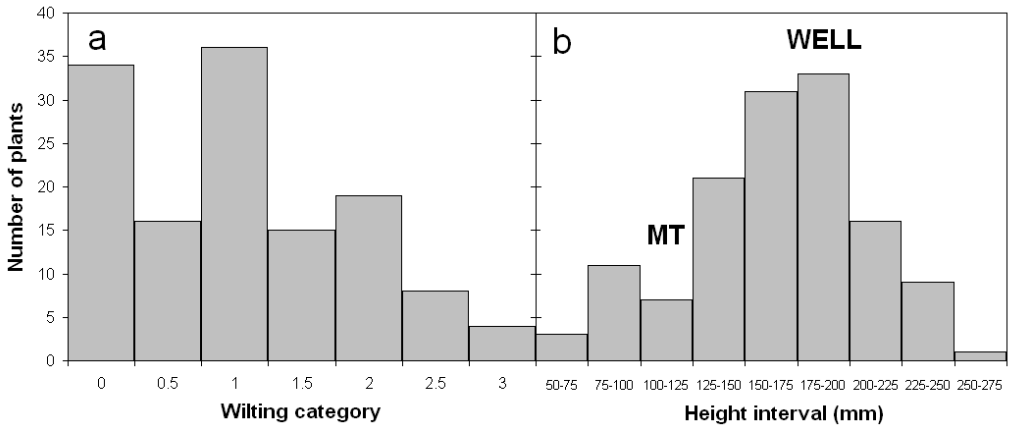

Figure 6. Experiment 1. Histograms showing (a) degree of wilting and (b) height in a segregating F_2 population derived from WELL x MT cross. Wilting was assessed in arbitrary units (0-3 scale; 0 most turgid, 3 most wilty) on 67-day old plants. Height was measured as the distance from the soil to the highest plant node. The intervals for the height of MT and WELL are shown. n=132

Wilting was assessed visually and by touch. Seven arbitrary categories (0, 0.5, 1…3) were used to assess this parameter. F_2 plants were found that could be scored as short and turgid or tall and wilty suggesting recombination between different genes controlling the two phenotypes (Fig 7).

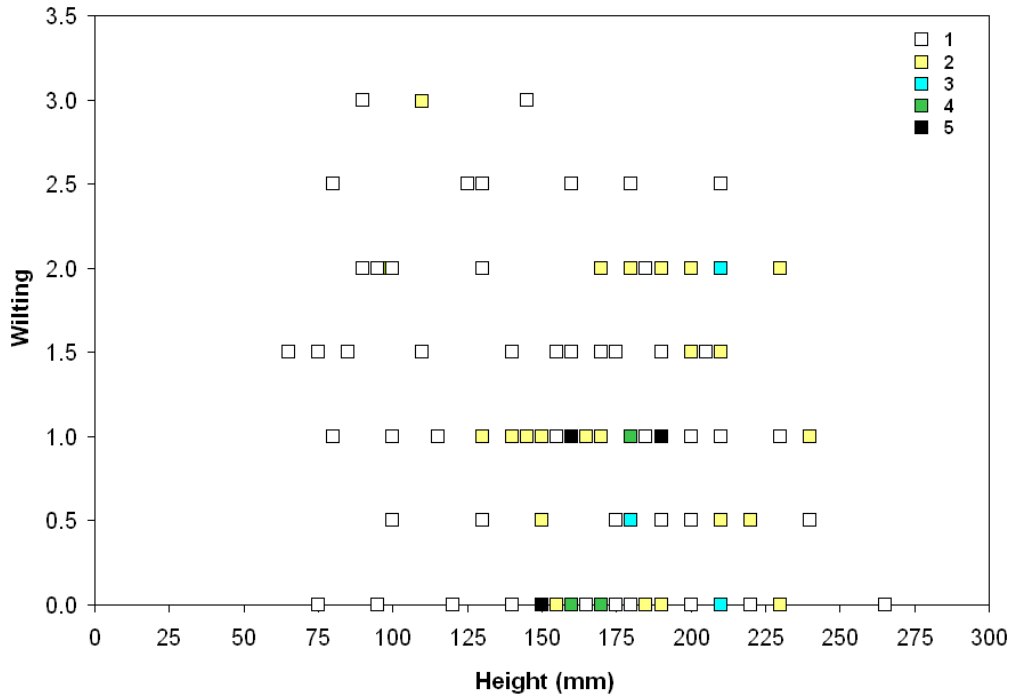

Figure 7. Scatter plot showing the relationship between height and wilting in a segregating population (n=132). Each dot represents an individual plant. Color code indicates number of overlapping individuals on the same wilting/height values.

Attempts were therefore made to fit the wilting data to a meaningful segeregation but this proved impossible *e.g.* molecular marker analysis suggested some short and turgid F2 plants such as plant #35 (Appendix 4E) did not carry any *S. pennellii* DNA. This suggests the need in future work for a more quantitative screen for delayed wilting, based for example on leaf temperature which, in a given environment correlates well to transpiration rate and on ^{18}O enrichment in leaf organic matter (Farquhar et al., 2007). Both characteristics can be measured relatively quickly and non -destructively and could be employed to reliably search for recombinants for height and wiltiness, before carrying out a more exhaustive analysis.

4.3.3. Generation of recombinant sub-lines with reduced introgression segments

A substantial range of phenotypic variation was observed among the F_2 plants. Segregation was noticed for traits such as plant height, number of leaves and fruits and total fruit fresh weight, shiny/glossy as opposed to matte appearence of leaves. To narrow down the *S. pennellii* regions responsible for these two phenotypes in WELL, a genetic screen of the F_2 population was conducted.

4.3.3.1. Identification and preliminary characterization of recombinants sub-lines of WELL

Total genomic DNA was extracted from all 132 F_2 individuals and genotyping was carried out using the markers described in the Methods section 4.2. Genotyping results are presented in Appendix 4E. Nine recombinants were found between markers C2_At1g14310 (30.5 cM) and C2_At2g45910 (73.0 cM). Additional recombinants may have been present and remained undetected but these nine recombinants, plus the recombinant identified using morphological markers, were deemed sufficient because they gave complete coverage of the original introgression by smaller, overlapping segments (Fig 8).

Interestingly, of the nine recombinants, those covering the region of the introgression close to the centromere (#45, #50) showed the tall phenotype characteristic of WELL (Fig 9, Table 4). Line #63 was not measured because this plant was attacked by spider mites, which caused stunted growth. Lines #41 and #130 were of an intermediate height between MT and WELL (Table 4). All of these plants were heterozygous for the recombinant *S. pennellii* fragment, so one more generation (F_3) had to be grown and genotyped to produce homozygous lines and multiply seeds, enabling their physiological evaluation.

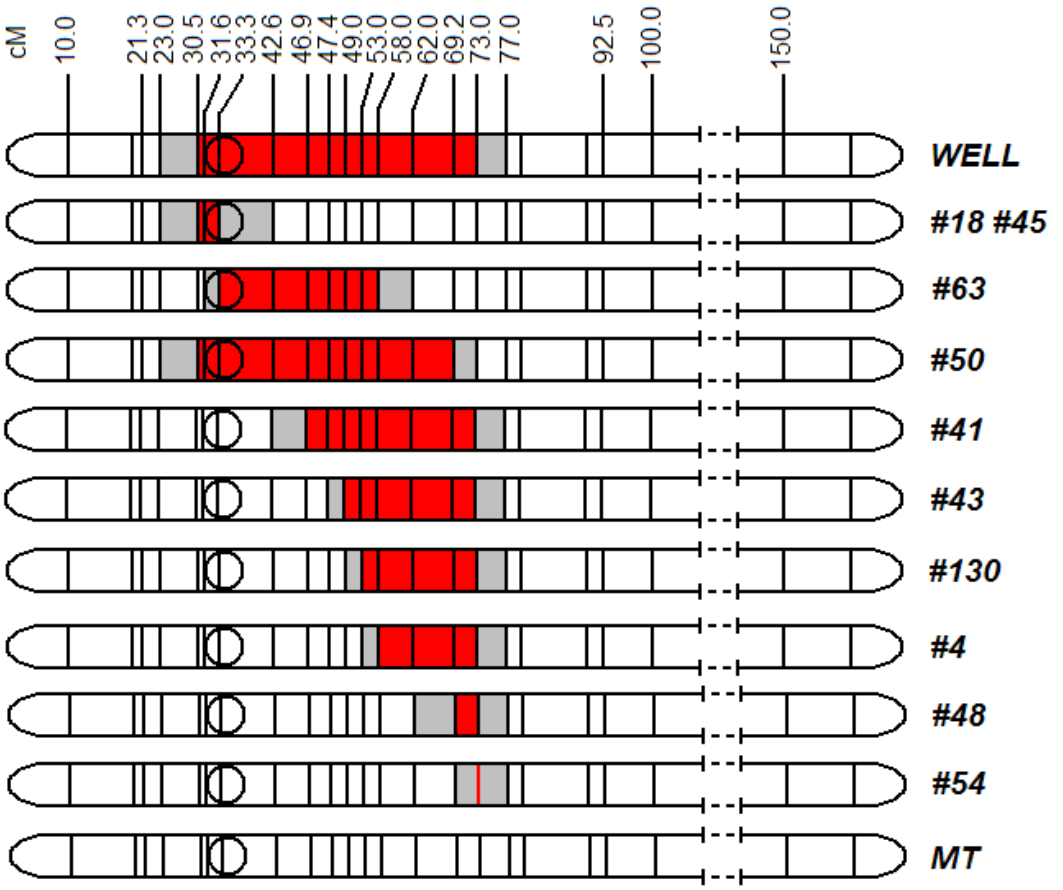

Figure 8. Experiment 1. Schematic representation of chrosomosome 1 depicting the *S. pennellii* introgression (red bars) in WELL and the 10 recombinant F_2 plants derived from WELL. Grey bars represent regions containing recombination break points. Marker names for each position are specified in Appendix 4C.

Table 4. Height of 62-day-old heterozygous F_2 recombinant plants. Values for MT and WELL are means of 10 replicate plants each.

Line	MT	WELL	#4	#18	#41	#43	#45	#48	#50	#54	#130
Height (mm)	115	180	145	180	150	130	210	130	160	75	145

Figure 9. Experiment 1. Growth phenotype of recombinant plants carrying smaller segments of the original *S. pennellii* introgression in WELL as described in Figure 8. In each panel: left, a representative MT control; right, F_2 individual. All plants are F_2 heterozygous recombinants identified using molecular markers, except sub-line #18 (top left), which is a homozygous line generated as described in section 4.3.1. Sixty two day-old plants grown in well-watered conditions.

4.3.3.2. Generation of homozygous recombinants

The recombinants identified in the segregating F_2 population (#4, #41, #43, #45, #48, #50, #54, #63 and #130) were all heterozygous for the *S. pennellii* fragments, so they were selfed and the next generation was genotyped to find homozygous individuals. The F_3 seeds were sown and thirty plants (or the closest possible number, depending on seed availability) of each F_3 family were cultivated in glasshouse (well-watered) conditions alongside MT and WELL controls. The segregation ratios for height confirmed what was observed in the previous generation: lines #45, #50 and #63 containing the long arm end of the original introgression all produced tall plants, in a roughly 3:1 (tall:short) ratio in all cases. The progeny of the other lines were all short, *i.e.* of similar height to MT.

The homozygous F_3 recombinants were transplanted to large pots and selfed to multiply seed. No homozygotes were found in the progeny of #50. Both #54 homozygous plants showed extremely low fertility and yielded only small, seedless fruits. In addition, the #63 plant identified as homozygous turned out to be heterozygous after a second round of genotyping was performed on all plants. Thus, this heterozygous individual was again selfed and its progeny (F_4) cultivated and screened again. Four homozygotes were then found out of 24 plants.

4.3.4. Physiological characterization of recombinant lines

Some of the recombinant lines found in the F_3 generation showed reduced fertility and had to be cultivated again or isolated from the progeny of heterozygous individuals of the previous generation. This limited availability of seed precluded growing all recombinant lines simultaneously in a large-scale experiment. Two successive experiments were thus performed as homozygous seeds became available, Experiment 2 comparing the drought response of line #54 and MT, and Experiment 3, comparing lines #4, #43, #45, #48, #63 and #130. Not enough seeds of line #41 were available at this time.

4.3.4.1. A recombinant line containing the long arm end fragment of the WELL introgression shows neither delayed wilting nor enhanced WUE

Experiment 2 was carried out to assess the response of line #54 to water withdrawal compared to MT. Setup and growth conditions are detailed in Appendix 4A and Methods section. Watering was suspended on 56-day-old plants, at a stage when the fifth leaf was beginning to unfurl in most plants. The first signs of wiltiness were evident 5 days later in the non-watered batch, with no apparent difference between genotypes. All plants were harvested after two more days. Leaf area and dry weight, whether

compared for the whole plant or individual leaves were similar between MT and #54 under well-watered conditions (Fig 10a, c). This confirms the prior observation that #54 was the recombinant line most closely resembling MT phenotypically. Although, as expected, leaf area was considerably reduced under drought, there was no difference between genotypes in this response (Fig 10a, b). Leaf dry weight suffered a concomitant reduction in both genotypes (Fig 10d). Specific leaf area (SLA), the amount of leaf area produced per unit dry weight, was also reduced to a similar extent for both genotypes (Table 5a).

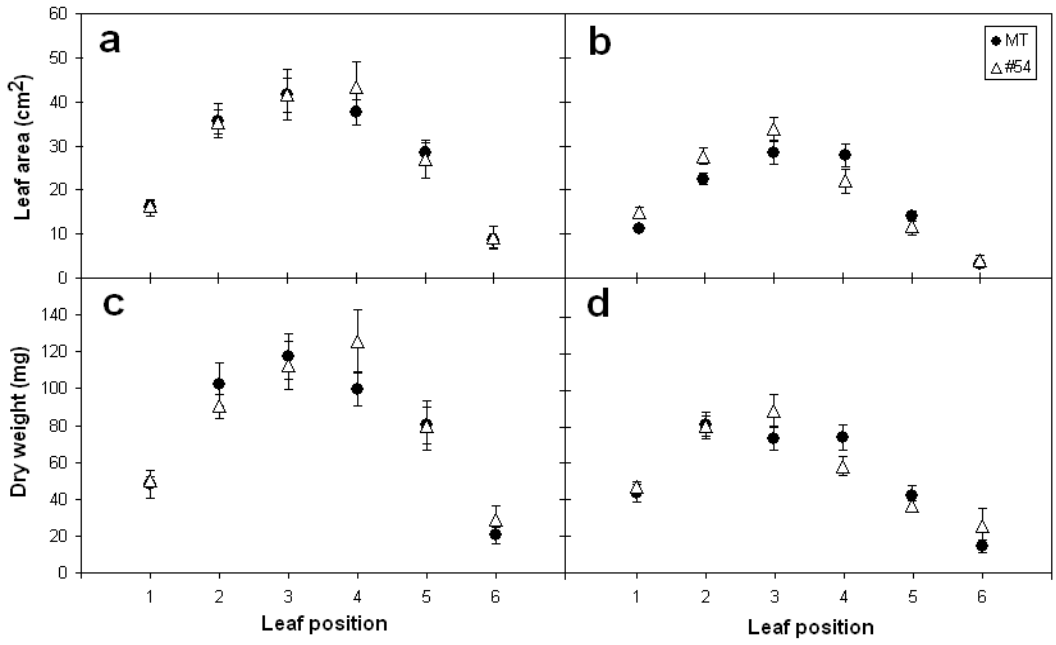

Figure 10. Experiment 2. Response to drought in line #54 compared to MT. Water was withdrawn for 7 days in 56-day-old flowering plants of similar leaf area. (a) Leaf area, well-watered. (b) Leaf area, no watering for 7 days. (c) Leaf dry mass, well-watered. (d) Leaf dry mass, no watering for 7 days. Mean values (n=7) ± s.e.m.

Carbon isotope discrimination ($\Delta^{13}C$) was determined for leaf six of both genotypes, which developed entirely after the beginning of the drought treatment. As usually observed under water limitation (Martin and Thorstenson, 1988), suspending watering for one week led to a significant decrease in $\Delta^{13}C$ (Table 5a) indicative of an increase in WUE. There was, however, no difference between genotypes for this parameter either (Table 5b).

Table 5a. Experiment 2. MT and #54 values of physiological parameters measured 60 days after germination; Aboveground (stem and leaves) dry weight; total leaf area (cm^2); and C isotope discrimination ($\Delta^{13}C$, measured on leaf 6) seven days after suspension of watering. Mean ± s.e.m. (n=6)

	MT		#54	
	Control	*Drought*	*Control*	*Drought*
Dry weight	779.19 ± 41.94	516.37 ± 27.69	798.88 ± 62.41	516.87 ± 25.39
Leaf area	168.07 ± 4.42	108.98 ± 4.66	172.1 ± 5.97	116.59 ± 2.67
$\Delta^{13}C$	21.00 ± 0.14	19.37 ± 0.29	21.07 ± 0.09	19.53 ± 0.24

Table 5b. *p*-values calculated with Tukey's HSD test for the parameters measured in Table 7a: ** indicates significant differences at $p < 0.001$

	MT	#54	MT v. #54	
	Control-Drought	*Control-Drought*	*Control*	*Drought*
Dry weight	0.0002**	0.0013**	0.7979	0.9896
Leaf area	0.0001**	0.0001**	0.5974	0.1819
$\Delta^{13}C$	0.0003**	0.0001**	0.6815	0.6783

4.3.4.2. The recombinant lines have different growth responses under drought

Experiment 3 was done to compare the response to drought between the recombinant lines and MT. The reduced fertility of lines containing the short arm end of the introgression (#18, #45) hampered the production of a sufficient number of seeds. Thus, line #18 could not be tested in this experiment and #45 was assessed with a reduced number of biological replicates (n=3), a cause for caution when analysing the results. The recombinant line #41 showed normal fertility but was genotyped and identified later, so not enough seeds were available at the time this experiment was conducted. As an initial comparison between lines, the ratio of values measured for droughted plants compared to control plants was calculated for each parameter (leaf area, leaf number, dry-weight, rate of transpiration etc, see Methods) for each genotype. A principal component analysis (PCA) was then conducted. After centering and normalising the data it was found that principal component 1 (PC1) had 45.7% of the signal energy (square of corresponding singular value normalised to sum of squares of all singular values), PC2 had 32%, and 22.3% was in higher order components not shown in the plot (Fig 11). The values shown in Table 6 are the coefficients for the first 2 components in terms of the original variables. The recombinant lines #43, #63 and #130 were clustered together with the MT control. Line #45 was considerably displaced along the PC1 axis and lines #4 and #48 along the PC2 axis. The three lines #4, #45 and #48 were next

examined in more detail in comparison to the four others to try to account for the differences.

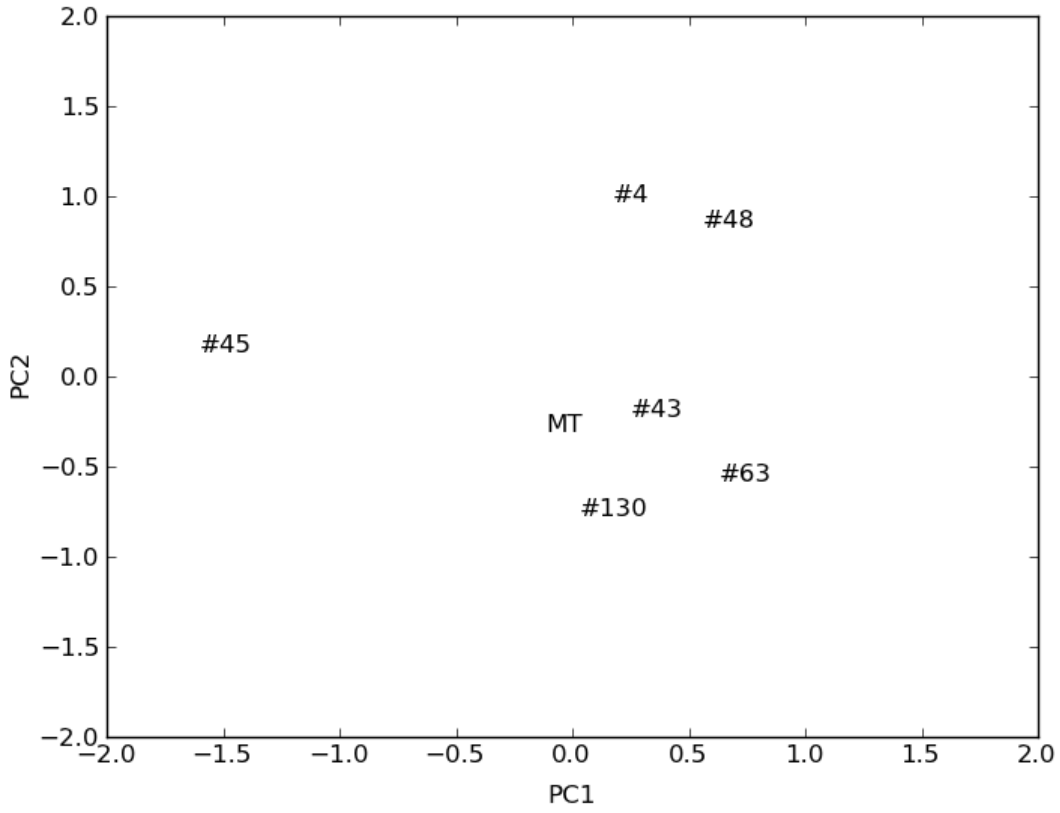

Figure 11. Experiment 3. Principal components analysis (PCA). Eight parameters, compared under well-watered and drought conditions, were analysed (Table 8). MT and selected recombinants were projected on the first 2 principal components. The first principal component (PC1) separates line #45 from the others, while the second principal component (PC2) shows a further separation of lines #4 and #48 from the main cluster.

Table 6. Experiment 3. Principal components analysis. Coefficients for the first 2 components in terms of the original variables. Every variable was measured as the ratio of the means between drought and control for each parameter for each genotype. Water loss (water transpired by plants over the seven-day drought period); *E* (transpiration, water lost per unit leaf area, measured at day 7 after start of drought treatment); SLA (specific leaf area, leaf area per unit leaf dry mass); LA (leaf area); leaf number and leaf dry mass (both measured at day 7).

Variable	PC1	PC2
Water loss	-0.496	0.169
E day 7	-0.433	0.128
SLA leaf 7	0.222	0.524
Leaf area days 0-7	0.275	0.0509
Leaf number day 7	0.234	0.476
SLA leaf 3	0.0738	0.545
E day 1	-0.330	0.388
Leaf dry mass day 7	0.520	-0.0272

The three recombinant lines grew normally under well-watered conditions, with more erect leaves and longer petioles than MT. Under well-watered conditions plants of line #45 had more leaves and were taller than the rest of the lines (Fig 12, Table 7). Increased height was also observed for line #4 (Table 7). Only plants of line #45 were significantly taller than MT under drought (Table 7).

Table 7. Average plant height (mm) of MT and homozygous recombinant lines under well-watered (WW) and drought (D) conditions. Mean ± s.e.m. (n=6). *p*-values calculated with Tukey's HSD test: * and ** indicate significant differences at p <0.05 and <0.001, respectively.

	MT	#4	#43	#45	#48	#63	#130
WW	74.2 ± 1.5	90.4±2.2 **	79.1 ± 2.0	97.0±3.4 **	73.4 ± 3.2	72.2 ± 1.9	77.8 ± 1.5
D	63.6 ± 2.4	66.2 ± 1.2	60.7 ± 1.3	71.6±1.6*	62.0 ± 1.9	58.2 ± 1.1	62.6 ± 2.0

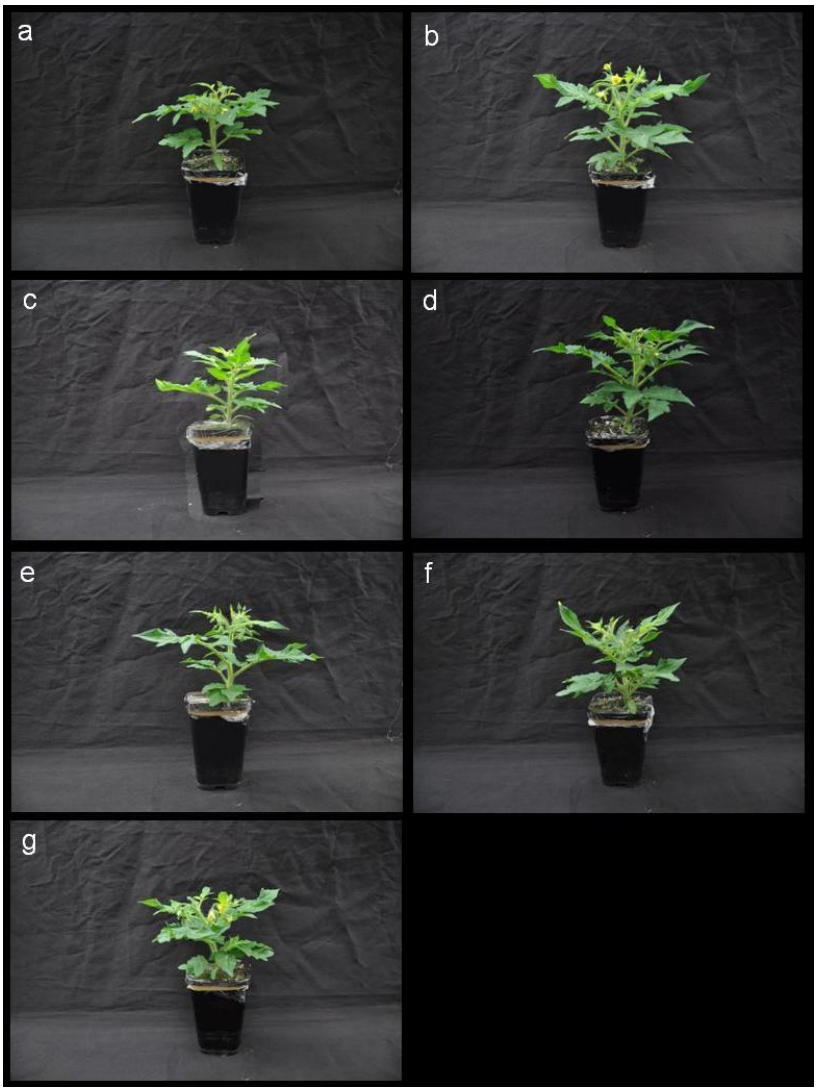

Figure 12. Experiment 3. Representative well-watered individuals of (a) MT, (b) #4, (c) #43, (d) #45, (e) #48, (f) #63 and (g) #130. Well-watered four-week old plants grown under controlled conditions in growth chamber (section 4.2.3).

The reduction in the total number of leaves under drought was not as severe in #43 (14% reduction) as in MT (22% lower), whereas line #63 had a reduced number of leaves under well-watered conditions but not under drought (Fig 13a). The relative leaf expansion rates between day 0 and day 7 of drought were not significantly different between lines within each treatment (Fig 13b). SLA was determined in two reference leaves for each genotype, leaf 3, which was fully-expanded (five leaflets present, elongated petioles) at the onset of drought, and leaf 7, which formed entirely during the seven days of drought (Fig 13c, d). SLA of leaf three was significantly higher in lines #45 and #130 under both well-watered and drought conditions (Fig 13c). Line #48 had higher SLA than MT under ample water supply, but lower under drought. For leaf seven, which developed entirely under conditions of water deprivation, the decrease in SLA was more severe for all genotypes (*e.g.* 46% for MT), there was a higher reduction (*i.e.* 'thicker' or denser leaves) in line #48 (55% lower). Line #130, on the other hand, showed less reduced SLA under drought (Fig. 13d).

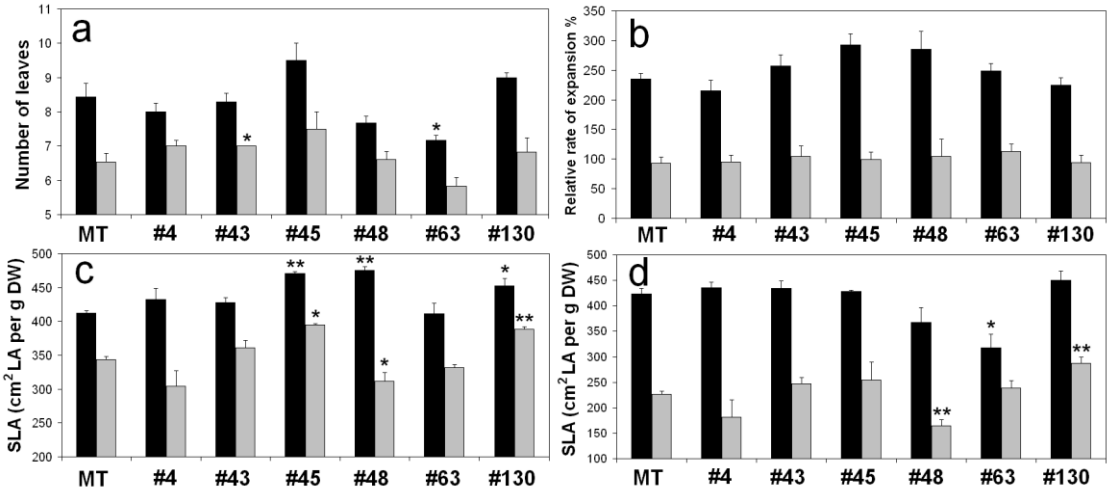

Figure 13. Experiment 3. Response to drought in MT and lines #4, #43, #45, #48, #63 and #130. (a) Number of primary leaves; (b) relative rate of leaf area expansion between days 0 and day 7 of water withdrawal (cm^2); specific leaf area (SLA) in (c) leaf 3 and (d) leaf 7, at the end of the drought period for well-watered (black bars) and drought (grey bars) treatments. Mean values for n=7 (except #45, n=3), bars represent s.e.m. *p*-values calculated with Student's *t*-test: * and ** indicate significant differences (to MT control, within treatments) at $p < 0.05$ and < 0.001, respectively.

The reduction in transpiration rate (*E*) in plants deprived of water compared to well-watered controls became evident on the fourth day after the start of the drought treatment, indicating the water available in the pots had been depleted to a limiting level (Fig 14). The general trend seemed to be that for all lines E started off decreasing by less than 15% per day, then by day four and day five a larger decrease of 20%-30% per day was observed compared to the well-watered control plants (Fig 14). the ratio of *E* in drought compared to control plants remained stable on day six, but it dropped again on day seven. There did not appear to be much difference between lines but this needs following up as there was a lot of noise and intra-line variability in the experiment. Further work, with a better experimental design, higher number of plant replicates and appropriate

statistical treatment, is required to draw inferences about the effect of each introgression on plant growth under conditions of reduced water availability in the soil(Table 8).

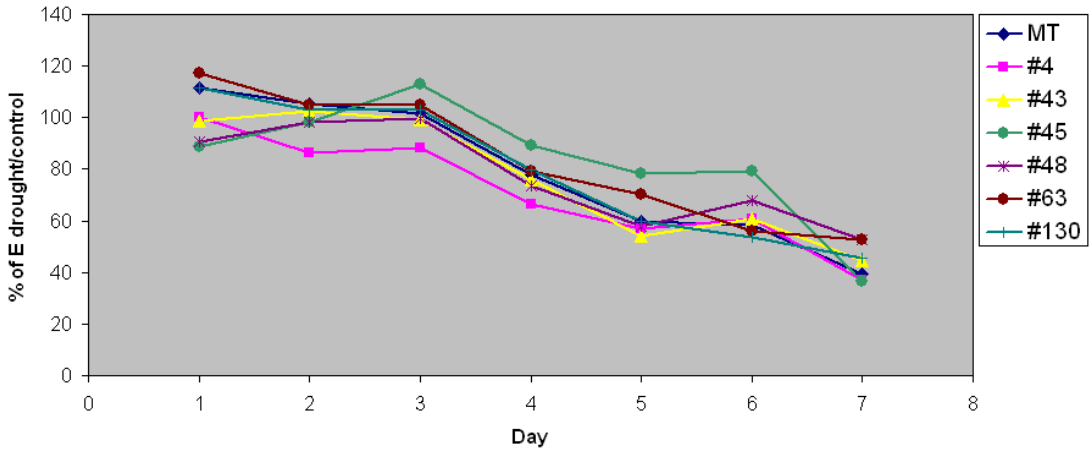

Figure 14. Experiment 3. Daily ratio of *E* under water restriction to *E* under well-watered conditions.

Table 8. Slope of E over the seven day period of water withdrawal (drought) compared to well-watered controls in MT and homozygous recombinant lines.

	MT	#4	#43	#45	#48	#63	#130
Control	-4.16	-3.83	-4.75	-4.88	-3.98	-3.38	-2.59
Drought	-12.40	-11.76	-11.93	-11.73	-10.72	-11.21	-11.58
Ratio D/C	2.98	3.07	2.51	2.40	2.69	3.31	4.46

4.4. Discussion

The work described in this chapter showed that the WELL introgression covers a large segment of tomato chromosome *1*, corresponding to a region of 42-54 centiMorgans (cM). The number of genes

can be estimated because of the strong correlation between genetic and physical distance in the tomato genome map (Tanksley et al., 1992), and over the ~950 cM of the tomato genome is, on average, 730 kb per cM (Tanksley et al., 1992). This ratio, however, can be locally reduced, owing to chromosome rearrangements that alter the recombination frequency so that estimates of genetic distance can vary by orders of magnitude (Ganal et al., 1990; Segal et al., 1992) A large number of chromosome re-arrangements have been reported for tomato relative to *S. pennellii* (Kamenetzky et al., 2010). Further, the *S. pennellii* genome is 20% larger than the tomato genome, so some meiotic pairing mismatch would be expected in interspecific crosses as a result of differences in chromosome length (Khush and Rick, 1963). Lastly, the introgression possibly encompasses the heterochromatic region in and around the centromere of chromosome *1* (Chang et al., 2007). The centromere is known to cause suppression of recombination in flanking sequences through a physical disruption of crossing-over caused by heterochromatin (Beadle, 1932; Roberts, 1965). This effect has also been demonstrated in interspecific crosses of tomato with *S. pennellii* (Khush and Rick, 1967; Rick, 1969). There are approximately 30,500 genes encoded in the euchromatic and 4,500 in the heterochromatic regions of the tomato genome, whereas the latter account for only one fourth of the genome's total size in base pairs (Peterson et al., 1996; Van der Hoeven et al., 2002). In a segment between 42 and 54 cM one would then expect to find a large number of genes, too large to use a candidate gene approach in an attempt to identify the gene(s) responsiblefor the observed WELL phenotypes. This

suggested that working with WELL would greatly hamper the effort to isolate a single genetic factor controlling wilting and/or WUE. Hence, a series of WELL recombinant lines with a reduced *S. pennellii* fragment was generated, which divided the original introgression into eight different, overlapping, chromosome bins. The aim of creating the set of recombinants was to see whether the different WELL phenotypes were genetically linked or not. Recombinant lines with a relatively small introgression size were retrieved for the outer regions of the introgression and larger ones for the centre. Whereas some lines still carried a large chromosome segment from *S. pennellii* (#63 and #41, both at least 24 cM), others were smaller (#18, #45, #4, #48) and one was potentially less than 1 cM (line #54). The phenotypic segregation of an F_2 population derived from the MT × WELL cross could not confirm whether the increased height segregated as a single Mendelian gene. Wilting is an extremely difficult character to quantify by visual inspection, and although attempts were made at standardizing its determination (Engelbrecht et al., 2007; Muir and Moyle, 2009), it was not possible to remove a component of subjectivity from the observations, nor to define clear discrete classes of leaf wiltiness. Measuring leaf turgor pressure or imaging leaf temperature may have provided more robust and unbiased albeit labour-intensive alternatives. Nevertheless, the fact that a great degree of variation in wilting was observed between individuals of a reasonably large segregating population (n=132) reinforced the idea that the introgression from *S. pennellii* improved drought response in the MT background. The reduction of the *S. pennellii* chromosome segment was achieved through a

combination of phenotypic (one recombinant line) and genotypic screens (nine recombinant lines) of the segregating F_2 population described above. Ten recombinant lines were found, that covered the original introgression in smaller overlapping chromosome segments. The seed multiplication process was held back by a series of difficulties related to plant culture, and ultimately, the time to conduct experiments characterising the recombinant lines was considerably reduced. In spite of this setback, some preliminary observations are discussed.

The recombinant line #54 was similar to MT in size, and contained a small fragment which, according to the initial release of the tomato genome sequence, harboured at the most ~100 genes (http://solgenomics.net/ genomes/Solanum_ lycopersicum/genome_data.pl). No difference was observed in its response to water withdrawal compared to MT. In contrast, differences in growth responses to water deprivation were observed among lines #43 (number of leaves) and #48 (SLA). Leaf samples were collected to determine stomatal densities and verify this, but results could not be included for lack of time within the timeframe of this thesis.

SLA was more reduced in line #48 than in MT, whether in leaves expanded before and during the drought period. SLA has often been observed to decrease under drought conditions (Marcelis et al., 1998). The differential decrease in SLA under drought conditions reflects the different sensitivity to soil drying of growth in mass compared to expansive growth.

Thicker or denser leaves usually have a higher density of chlorophyll and proteins per unit leaf area and, hence, have a greater photosynthetic capacity than thinner leaves. Samples will be analysed for carbon isotope composition to examine this.

The data obtained in the sub-recombinants are preliminary and await consolidation through additional measurements on the samples collected as well as further experiments. The results presented here are not consistent with a single line having the drought response observed in WELL, so further analyses should be conducted. Line #4 carries a segment of at least 15 cM in the long arm end of the original WELL introgression (58-73 cM, Fig 8) and #48 a shorter overlapping bit of at least 4 cM (69-73 cM). Although the *S. pennelli* fragment in lines #43, #48 and #130 also span this region a 4 cM gap (73-77 cM) of unknown genotype flanks the more distal recombination point of all three lines, so it is possible that #4 and #48 carry genes from *S. The* development of PCR markers to increase the resolution of the genotyping as well as more detailed physiological experiments focused on stomatal dynamics and development in relation to plant water status will clarify this issue.

Chapter 5 – Conclusions and future directions

5.1. Conclusions

5.2. Future directions

5.1. Conclusions

Agriculture is the biggest consumer of fresh water in the world. Boosting plant yield using the same amount of water and increasing the resistance of crops to drought, are two of the greatest challenges of plant biology for the 21st century. Tomato (*Solanum lycopersicum* L.) is an excellent genetic model with a rich source of natural variation in its wild relatives of the genus Lycopersicon (now genus *Solanum* section *Lycopersicum*). Among them, *S. pennellii* Correll, is adapted to arid conditions and exhibits a remarkable tolerance to water deficit in the soil and a high water-use efficiency (WUE) measured as biomass gained per water lost. A series of crosses and screening steps were performed with the aim of introducing some genetic material for conferring increased drought resistance from *S. pennellii* into the miniature cultivar Micro-Tom, an amenable model system for tomato genetics.

S. pennellii (LA716) was crossed to *S. lycopersicum* cv Micro-Tom (MT) and the F_1 was selfed and the resulting F_2 seedlings were screened for reduced height. Water was withdrawn from pots with control and hybrid plants, and these were scored visually for wilting symptoms as compared to the MT control in the same pot. F_2 individuals showing delayed wilting were rewatered and seed harvested. This progeny was grown and back-crossed to MT at least 5 times (BC_5). A homozygous line was retrieved in BC_5F_3 which, although dwarf as the recurrent parent MT, showed increased height, semi-

determinate growth habit, more erect leaves, and delayed wilting upon the suspension of watering. WUE was determined for MT and the introgression line by gravimetry, carbon isotope composition and gas exchange. No physiological difference was observed between genotypes before flowering, but the introgression line showed a higher WUE than MT after flowering. The line was named WELL for Water Economy Locus in Lycopersicon. As discussed in Chapters 1 and 3, the mechanisms for improved drought resistance are not reduced to mechanisms giving increased WUE. For example, it could have been that the line herein recovered, with delayed wilting, would not have had increased WUE, if *A* decreased in proportion to g_s, which would not be unexpected, or respiratory losses were increased . If this were the case, it would still be quite an interesting line, as maintaining cellular turgor is essential to maintaining cell biochemistry and preventing death; also essential with respect to fertilisation and fruit set

MT and WELL plants differed considerably in stomatal conductance (g_s) when grown together in the same pot under conditions of reduced water availability, with WELL having more reduced g_s values than MT. WELL also had delayed wilting in young seedlings of the same leaf area as MT. The consistent difference in g_s between genotypes suggests different stomatal sensitivity to water stress. Differences in stomatal dynamics may explain the behaviour of the two lines under drought, unless WELL has lower stomatal density. Anatomical features of leaves were analysed by microscopy to determine this. No significant differences were observed in stomatal density,

though there was a trend towards increased densities in WELL on the adaxial (upper) side of the leaf.

Genetic mapping using the chromosome *1* morphological markers *y* (colourless fruit epidermis, flavonoid pigment absent) and *sit* (wilty plants, ABA deficiency) suggested that the introgression in WELL was located on chromosome *1*. Genotyping with PCR-based markers confirmed this and allowed to estimate the size of the introgression at between 42 and 54 cM. This large portion of *S. pennellii* genome could harbour thousands of genes. This hinted the possibility of producing sub-lines of WELL with reduced introgression sizes which would in turn inform whether the multiple traits altered in WELL compared to MT could be separated via recombination. A segregating F_2 population was sown and genotyped using molecular markers for chromosome *1*. Its analysis indicated that the two characteristics, height and wilting, are unlikely to be causally linked. Hence, at least two different loci should account for each of this traits in WELL.

Ten recombinants with reduced introgression sizes were obtained, ranging from 24 cM (half the original size) to less than 1 cM. This set of overlapping recombinants covered the whole of the original *S. pennellii* introgression in WELL. Three of them showed persistent fertility problems and only produced very limited numbers of seeds. Interestingly, all three of these partially sterile lines contain overlapping introgressed segments on the short arm side of the original WELL introgression. The other seven

recombinants were characterised in their growth response under water deprivation. Two lines were retrieved, containing overlapping segments of the introgression in chromosome *1*, which showed slightly better growth under water deprivation. A more in-depth analysis of these lines should confirm whether any of them carries a gene or genes from *S. pennellii* conferring increased drought resistance.

5.2. Future directions

The promising results obtained in this work warrant further investigation of the genetic basis of delayed wilting and increased WUE in WELL. The recombinant lines will be characterised more in-depth and carbon isotope discrimination determined under well-watered and drought conditions to determine the approximante location of WELL in chromosome *1*. Fine mapping will be performed through the development of new markers to narrow down the position of the gene(s) controlling the stomatal response in WELL. It is appealing to discover whether a single genetic locus is responsible for the drought resistance in WELL, in which case the recombinant lines could be used as a starting point for positional cloning by candidate gene approach. It will also be of interest to explore the semi-determinate growth habit observed in WELL, since none of the genes described so far for this trait map on chromosome *1*. The relationship between growth habit and WUE is still far from clear and can be approached using this and other genotypes in the convenient MT genetic background,

where genes of interest can be readily introgressed. Lastly, a role for plant hormones in some of the traits observed in WELL should be investigated. If this is confirmed, it could pave the way for the characterisation of novel *S. pennellii* genes and/or alleles involved in hormonal pathways. Natural genetic variation for hormone sensitivity and biosynthesis is not a thoroughly explored issue and could yield valuable information for the understanding of the adaptation to drought in nature.

Appendices

3A –Experiments presented in Chapter 3

All experiments were carried out at the Australian National University in Canberra, Australia (35°18' S; 149°07'E; 559 meters above sea level). The conditions and setup of each experiment described on chapter 4 are detailed below. Canberra daylength over the course of the year is shown in Fig 4A1. The monthly average hours of sunlight after taking into account cloud cover, as well as average ambient relative humidity, is shown on Table 4A1.

Experiment 1

Date: May-July 2008

Setup: MT and WELL seeds were surface-sterilized by treatment with a 5% (v/v) solution of household bleach (White King, Australia) for 5 minutes and then washed in distilled water. Seeds were then germinated on wet filter paper in Petri dishes. Two weeks after germination seedlings were transplanted to 3L pots filled with 3.5 kg of pasteurised sand. After watering and leaving pots to drain to 'field capacity', pots were wrapped in plastic foil to prevent evaporation from the soil and weighed 'initial weight'. Watered every second day with 1/3 Hoagland's solution, every time topping up to the initial weight and recording pot weight. Harvested twice, 30 and 50 days after germination. Whole plants were harvested and fresh weight determined

after thorough washing of roots. The fresh material was subsequently oven-dried for 48h at 70°C and then dry weight determined. Gas exchange measurements were performed on a third batch at 60 days after germination.

Environment: glasshouse

Irradiance: around 500 μmol m^{-2} s^{-1} (average) with peaks of 800 μmol m^{-2} s^{-1} at midday

Temperature: 26°C/20°C day/night

Relative humidity: 20/40% day/night

Experiment 2

Date: June-August 2008

Setup: MT and MT *SP*/*SP* seeds were sterilized and germinated on wet filter paper in Petri dishes. Two weeks after germination seedlings were transplanted to 1.7L pots filled with 2.2 kg of pasteurised sand. 25 days after germination pots were flushed with water and when dripping from the bottom had stopped, pots were wrapped in plastic foil to prevent evaporation from the soil and weighed 'initial weight'. Pots were watered every second day with 1/3 Hoagland's solution, every time topping up to the initial weight and recording pot weight. Plants were harvested 60 days after germination. All plant dry material, shoot and roots (washed from the soil) was oven-dried for 48 hours at 70°C.

Environment: glasshouse

Irradiance: around 500 μmol m^{-2} s^{-1} (average) with peaks of 800 μmol m^{-2} s^{-1} at midday

Temperature: 26°C/20°C day/night

Relative humidity: 20/40% day/night

Experiment 3

Date: July-September 2008

Setup: MT and WELL seeds were sterilized and germinated on wet filter paper in Petri dishes, two weeks after germination, seedlings were transplanted in pairs (one WELL and one MT seedling per pot) to 1.7L pots filled with 2.2L of pasteurised sand. Pots were watered daily alternating between water and full-strength Hoagland's solution. At the onset of flowering (40 days after germination), half the pots ('drought' treatment) were watered with only half the amount of water/solution as the control batch.

Environment: glasshouse

Irradiance: around 500 μmol m^{-2} s^{-1} (average) with peaks of 800 μmol m^{-2} s^{-1} at midday

Temperature: 26°C/20°C day/night

Relative humidity: 20/40% day/night

Experiment 4

Date: November 2009-January 2010

Setup: Micro-Tom and WELL seeds were sterilized and germinated on wet filter paper in Petri dishes and transplanted to 1.7L pots with 2.2L of pasteurised sand.

Environment: growth chamber

Irradiance: 600 μmol m^{-2} s^{-1}

Temperature: 25/20°C day/night

Relative humidity: 60%

Experiment 5

Date: July-September 2010

Setup: Micro-Tom and WELL seeds were sterilized and sown in 40x20x5 cm trays filled with seed raising mix. Two-week old seedlings were transplanted to 0.15L pots filled with a 1:1 mixture of seed raising mix (Debco, Australia) and vermiculite (Ausperl, Australia) supplemented with 1 g L^{-1} 10:10:10 NPK (Scotts, Australia) and 4 g L^{-1} lime. Watering was done by sub-irrigation placing the pots inside trays with 4-5 cm of water. After allowing 2 days for seedling establishment and recovery from transplantation, water was removed from trays containing half the MT and WELL pots ('drought'). The other was kept in trays water was topped daily to the 4-5 cm mark ('control'). to minimise the risk of bias in allocating seedlings to the two treatments, the leaf area of all seedlings at the start of the experiment was estimated through measurements of leaflets' maximum width and length, seedlings were carefully selected according to leaf area, and care was taken that the average

value of total leaf area thus estimated was similar for the two batches of plants.

Environment: growth chamber

Irradiance: 500-800 μmol photons m^{-2} s^{-1} PAR

Temperature: 25/20°C day/night

Relative humidity: 60-75% day/night

Experiment 6

Date: January-February 2011

Setup: MT and WELL seeds were sterilized and sown in 40x20x5 cm trays filled with seed raising mix. Seedlings were transplanted to 1.7L pots filled with 2.2L of pasteurized sand.

Environment: growth chamber

Irradiance: 350 μmol m^{-2} s^{-1}

Temperature: 25/20°C day/night

Relative humidity: 60%

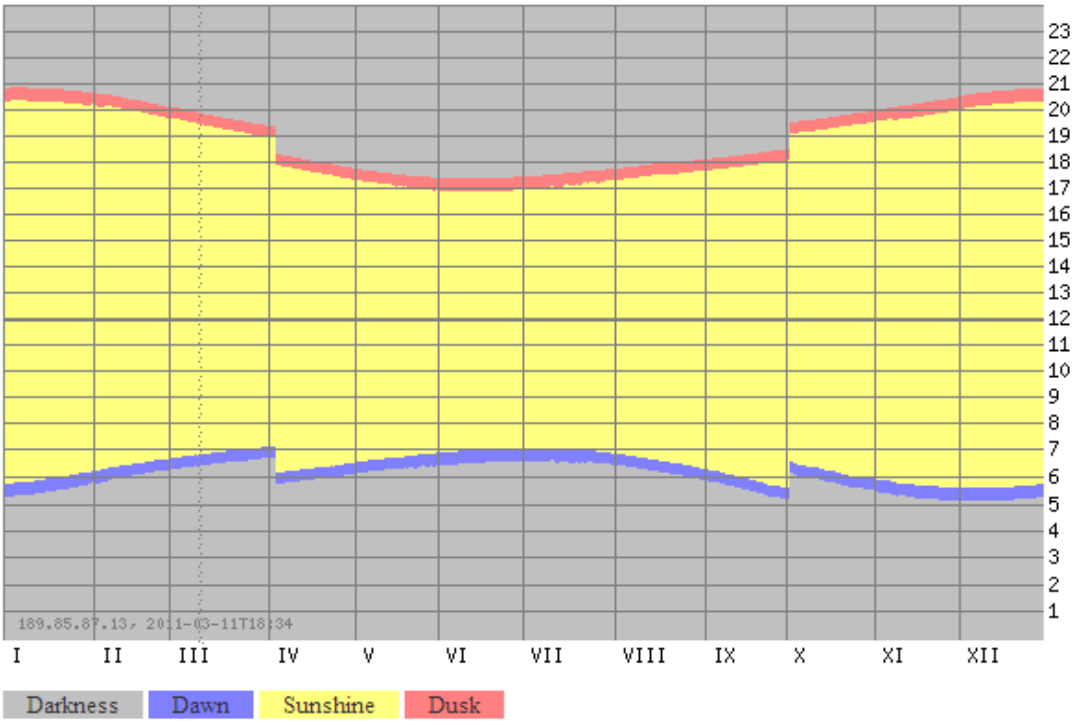

Figure 3A1. Graph depicting sunrise, sunset, dawn and dusk times in Canberra, Australia. The x-axis shows months of the year and the y-axis time of day in a 24-hour clock.

Table 3A1. Average hours of sunlight per day (Hs.) and average relative humidity (RH%) in Canberra, Australia.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Hs.	9.1	8.4	7.5	6.9	5.5	4.6	5.1	6.1	7.5	8.0	8.9	9.0
RH%	35	38	41	51	57	64	62	57	50	43	40	35

3B – Micrographs

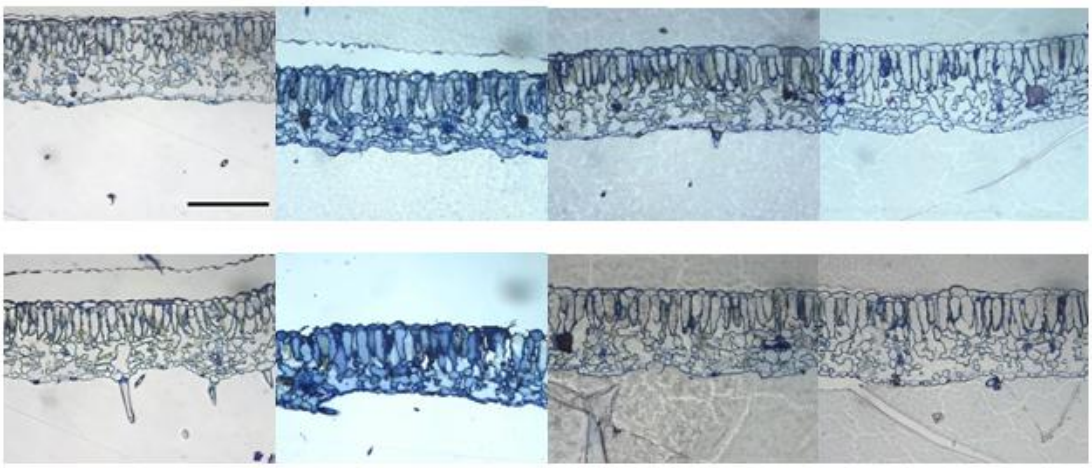

Fig 3B1. Cross-sections of mature MT leaves. Images on the same column represent different fields of view for the same sample. Scale bar = 100 µm

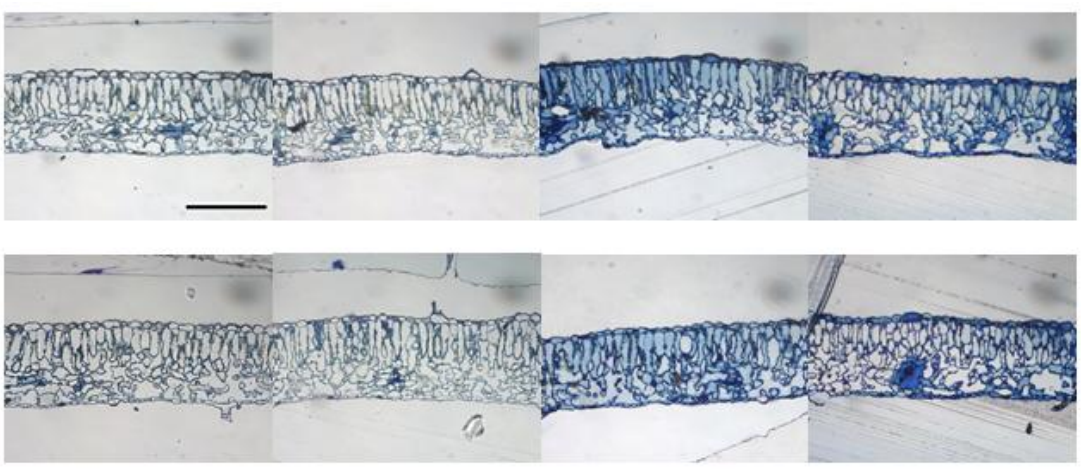

Fig 3B2. Cross-section of mature WELL leaves. Images on the same column represent different fields of view for the same sample. Scale bar = 100 µm

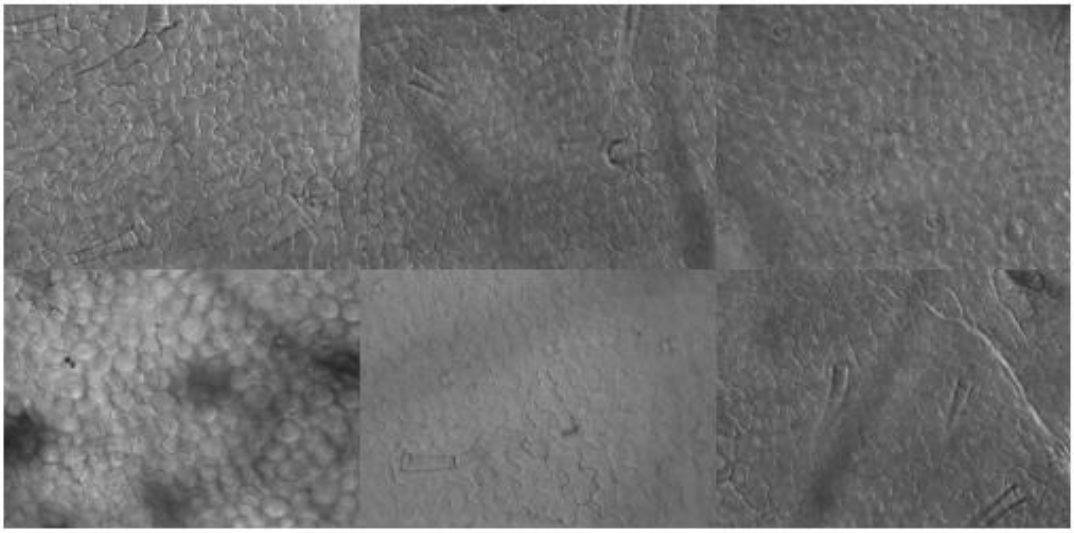

Fig 3B3. Micrographs of the adaxial surface of cleared mature MT leaves. Scale bar = 100 µm

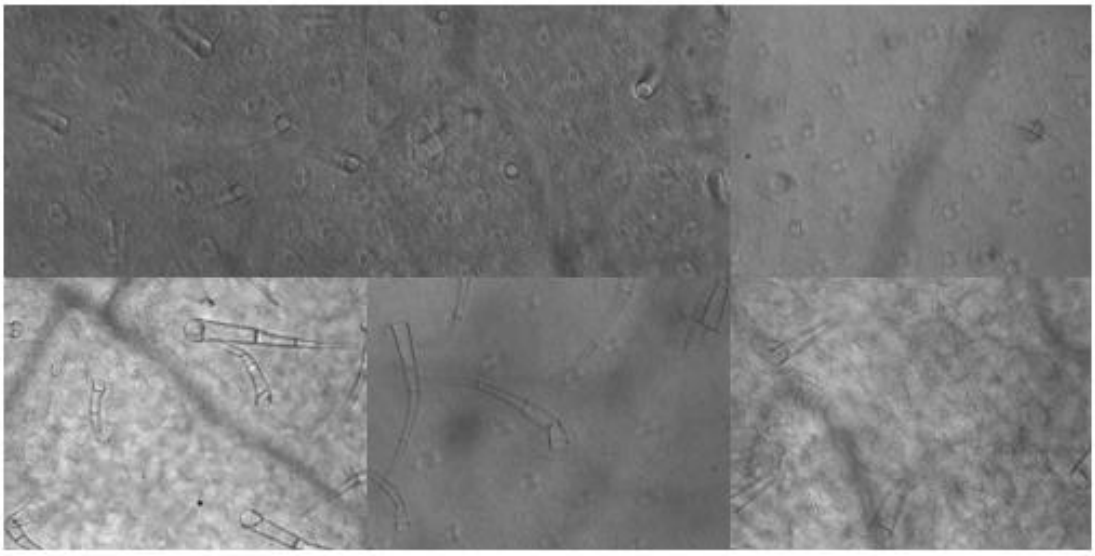

Fig 3B4. Micrographs of the abaxial surface of cleared mature MT leaves. Scale bar = 100 µm

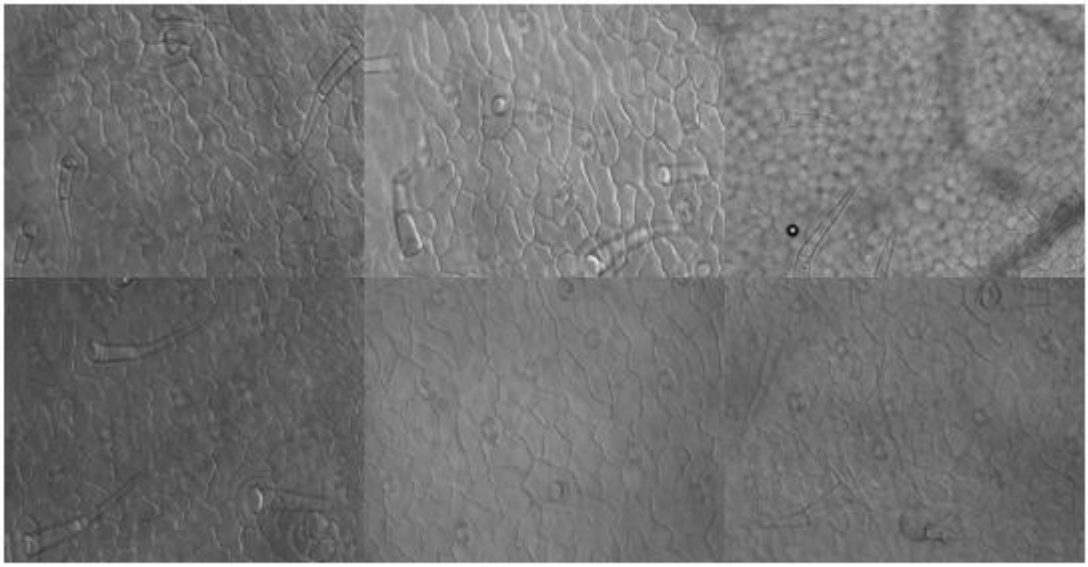

Fig 3B5. Micrographs of the adaxial surface of cleared mature WELL leaves. Scale bar = 100 μm

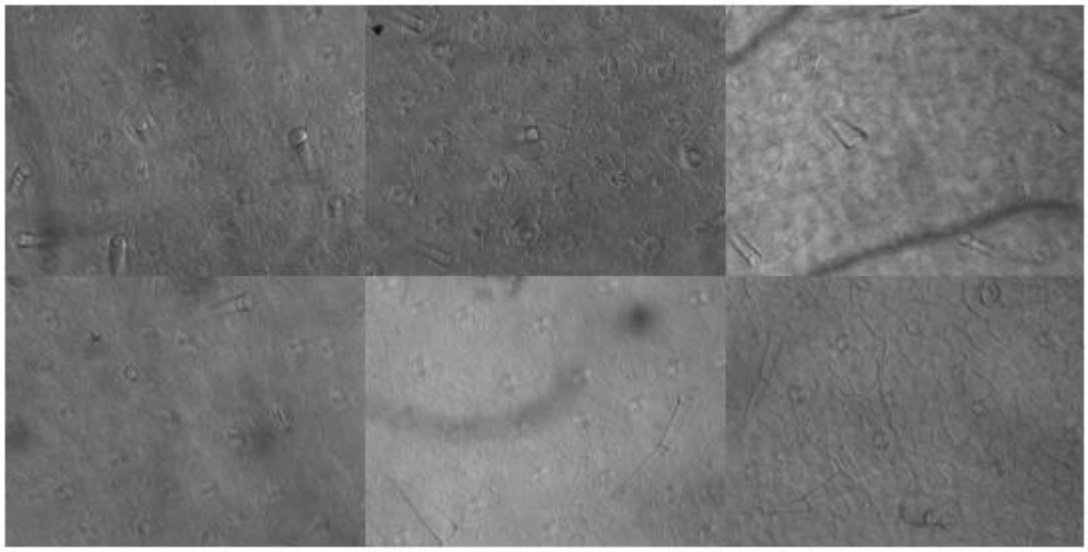

Fig 3B6. Micrographs of the abaxial surface of cleared mature WELL leaves. Scale bar = 100 μm

3C – Stomata and trichome densities

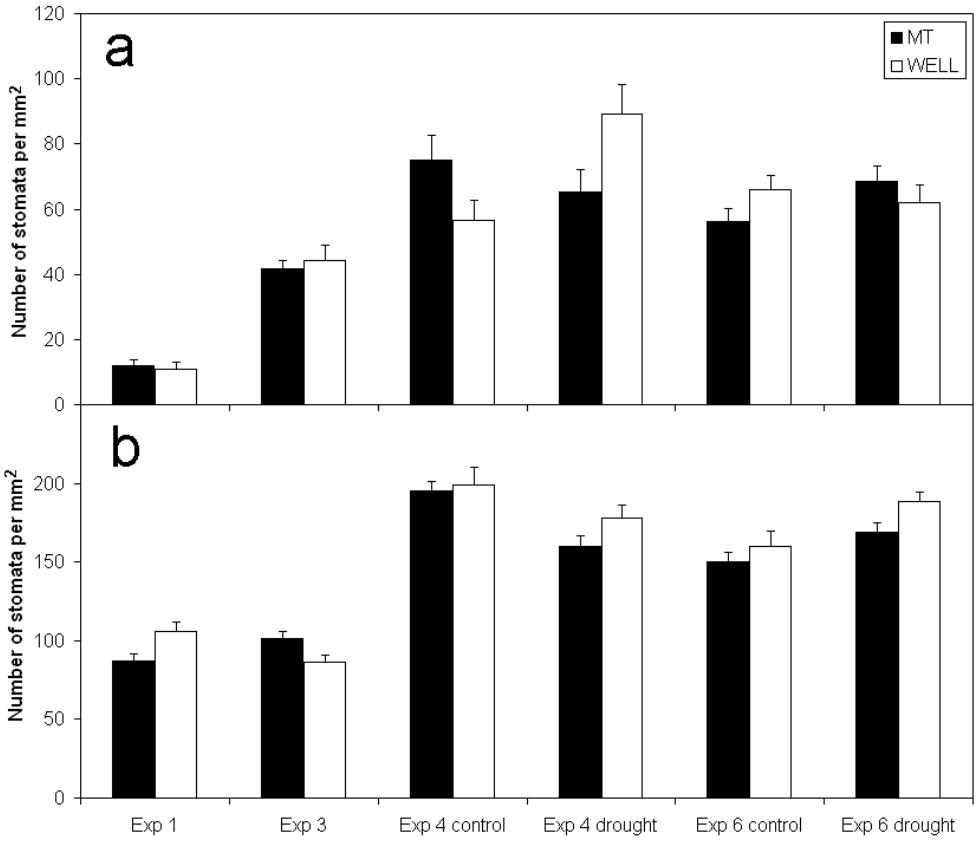

Fig 3B7. Stomatal density in the (a) adaxial and (b) abadial side of mature, well-exposed leaves of MT (full bars) and WELL (open bars) in experiments presented in Chapter 3.

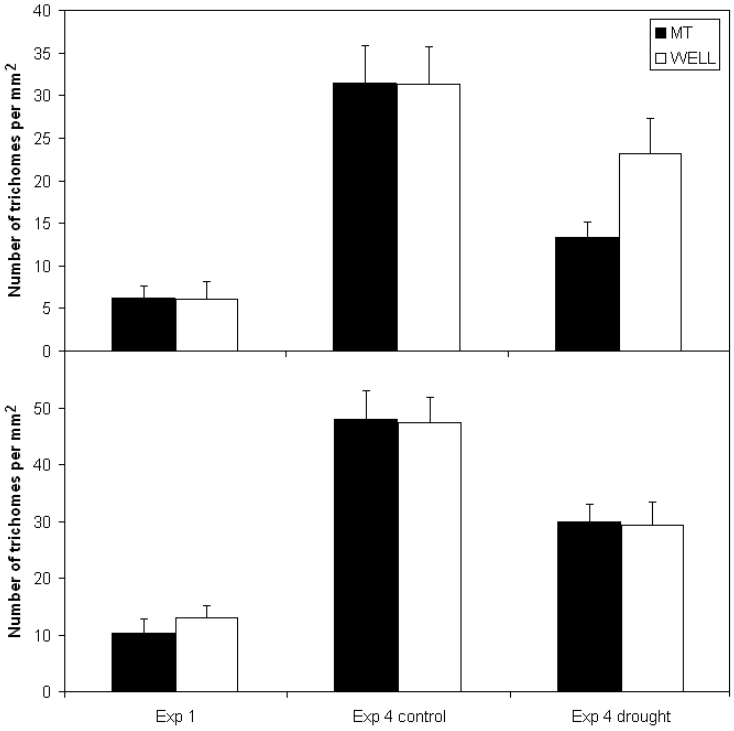

Fig 3B8. Trichome density in the (a) adaxial and (b) abadial side of mature, well-exposed leaves of MT (full bars) and WELL (open bars) in experiments presented in Chapter 3.

3D – Fruit yield and brix

Table 3D. Fruit traits of MT and WELL. Mean ± s.e.m. (n=6). *p*-value calculated with a *t* test: * and ** indicate significant differences at $p < 0.05$ and $p < 0.01$ respectively

	Condition	MT	WELL	*p*
Fruit number	Control	6.17 ± 0.74	9.67 ± 1.25	0.0367 *
	Drought	4.5 ± 0.67	10.5 ± 1.97	0.5811
Yield (fruit fresh weight, g)	Control	18.8 ± 2.24	15 ± 1.51	0.1898
	Drought	13.77 ± 2.62	13 ± 2.45	0.8343
Total soluble solids (Brix grades)	Control	4.63 ± 0.58	7.77 ± 0.76	0.0082 **
	Drought	5.6 ± 0.23	8.28 ± 0.35	0.0001 **
Yield x Brix	Control	88.47 ± 17.37	113.63 ± 10.00	0.2379
	Drought	78.88 ± 16.77	106.45 ± 19.63	0.3107

4A – Drought experiments presented in Chapter 4

All experiments were carried out at the Australian National University in Canberra, Australia (35°18' S; 149°07'E; 559 meters above sea level). The conditions and setup of each experiment described on chapter 5 are detailed below. Canberra daylength over the course of the year is shown on Appendix 3 (Fig 3A1). The monthly average hours of sunlight after taking into account cloud cover, as well as average ambient relative humidity, is shown on Appendix 3 (Table 3A1).

Experiment 1

Date: June-September 2009

Setup: Approximately 180 seeds of an F_2 population derived from the cross MT x WELL were surface-sterilized by treatment with a 5% (v/v) solution of household bleach (White King, Australia) for 5 minutes and then washed in distilled water. Seeds were sown in 40x20x5 cm trays filled with seed raising mix (Debco, Australia). Two-week old seedlings were transplanted to 0.3 L pots filled with a 1:1 mixture of seed raising mix and vermiculite (Ausperl, Australia) supplemented with 1 g L^{-1} 10:10:10 NPK (Scotts, Australia) and 4 g L^{-1} lime. Watering was done by sub-irrigation placing the pots inside trays with 4-5 cm of water. 75 days after germination watering was suspended and all water removed from the trays. After 5 days, wilting was evaluated visually as explained in the methods section of chapter 5.

Environment: glasshouse

Irradiance: around 500 µmol m^{-2} s^{-1} (average) with peaks of 800 µmol m^{-2} s^{-1} at midday

Temperature: 25°C/20°C day/night

Relative humidity: 60/75% day/night

Experiment 2

Date: August-October 2010

Setup: MT and #54 seeds were sterilized and sown in 40x20x5 cm trays filled with seed raising mix. Two-week old seedlings were transplanted to 0.3L pots filled with a 1:1 mixture of seed raising mix and vermiculite supplemented with 1 g L^{-1} 10:10:10 NPK and 4 g L^{-1} lime. Watering was done place by sub-irrigation placing the pots inside trays with 4-5 cm of water. Twenty-two days after seed germination, when the fifth leaf was beginning to unfurl in most plants, the leaf area of each plant was estimated measuring the length and width of each leaflet and adding up their products. Two batches were created for each genotype, each with a similar average value of leaf area. One of the batches was kept well-watered and the other was transferred to empty trays were water was withheld for a period of seven days, over which wilting was assessed daily visually and by touch. At the end of this period, plants were re-watered and their leaf area determined. All the aboveground plant material was oven-dried at 70°C for 48h and then weighed to 0.001 g in an analytical balance.

Environment: glasshouse

Irradiance: around 600 μmol m^{-2} s^{-1} (average) with peaks of 1000 μmol m^{-2} s^{-1} at midday

Temperature: 26°C/20°C day/night

Relative humidity: 40/60% day/night

Experiment 3

Date: October-December 2010

Setup: Seeds of MT, #4, #43, #45, #48, #63 and #130 were sterilized and sown in 40x20x5 cm trays filled with seed raising mix. Ten days after germination, seedlings were transplanted to 0.3L pots filled with a 1:1 mixture of seed raising mix and vermiculite supplemented with 1 g L^{-1} 10:10:10 NPK and 4 g L^{-1} lime. 16 replicate plants were grown per genotype, except for line #45 where there were six replicates. Watering was done place by sub-irrigation placing the pots inside trays with 4-5 cm of water. Twenty days after germination, the leaf area of all seedlings was estimated through measurements of leaflets' maximum width and length. The plants of each genotype were thus split in two treatments, each group having a similar average value of total leaf area (n=8 per treatment, except for #45, where n=3). Water was removed from all the trays containing the pots and pots allocated to two treatments: well-watered and drought. All pots were wrapped in plastic wrap and weighed at this point, and then daily over the duration of the experiment. Pots in the well-watered treatment were watered daily to the original weight, whereas no water was added to the 'drought' treatment pots. The same was done for empty control pots with soil but no plants. After seven days, leaf area was again estimated through measurements of leaflets' maximum width and length and then harvested and measured again using a LI-3050A Conveyer Leaf Area Meter (Li-Cor,

Nebraska, USA). Leaves were harvested individually in paper bags and oven-dried for 48h at 70°C, then weighed to 0.001 g in an analytical balance.

Environment: growth chamber

Irradiance: 350 μmol m^{-2} s^{-1}

Temperature: 25/20°C day/night

Relative humidity: 60%

4B – Estimation of genetic distances based on phenotypic frequencies

There are several methods to investigate potential linkage between genetic loci (Crow, 1990). The most commonly used methods are the maximum likelihood (Haldane, 1919), least squares, square-root (Kuspira and Bhambhani, 1984) and product ratio (Fisher and Balmukand, 1928).

When two genetic loci are linked, the distance between them can be estimated using the phenotypic ratios of a segregating F_2 population. The phenotypic ratio of recombinant to parental types results in a ratio (product ratio) which is a function of the recombination frequency. Calculating the recombination frequency allows to estimate the distance between loci.

Consider two autosomal loci A and B with complete dominance. Each locus has a dominant allele (A and B) and a recessive allele (a and b). If both dominant alleles are located in one homologue chromosome and both recessive alleles in the other, the genes are said to be in coupling phase (or *cis*). On the other hand, if each homologous chromosome has the dominant allele from one gene and the recessive one from the other, the disposition is called repulsion (or *trans*).

For a cross between two homozygote individuals with the genes A and B in ***coupling*** phase, with recombination frequency **r**, the gametic frequencies will be:

Gamete	AB	Ab	aB	ab
Frequency	½- ½ r	½ r	½ r	½- ½ r

The F_2 population is generated by selfing the F_1. The genotypic array in F_2 is obtained by multiplying the gametic array in females by the gametic array in males. This gives:

Genotype	Frequency
AABB	$\frac{1}{4} - \frac{1}{2} r + \frac{1}{4} r^2$
AABb	$\frac{1}{2} r - \frac{1}{2} r^2$
AAbb	$\frac{1}{4} r^2$
AaBB	$\frac{1}{2} r - \frac{1}{2} r^2$
AaBb	$\frac{1}{2} - r + r^2$
Aabb	$\frac{1}{2} r - \frac{1}{2} r^2$
aaBB	$\frac{1}{4} r^2$
aaBb	$\frac{1}{2} r - \frac{1}{2} r^2$
aabb	$\frac{1}{4} - \frac{1}{2} r + \frac{1}{4} r^2$

The recombination rate r can vary from 0.5 (independent assortment) to 0 (complete linkage). If there is no recombination, the genes are inherited as a single unit. If there is incomplete linkage, all genotypes are seen but there is a significant deviation from the genotypic array seen in independent assortment.

The phenotypic array can be obtained by adding up the appropriate genotypes:

Phenotype	Haplotype	Frequency
AB	Parental	¼ 2 + (1 - r)²
Ab	Recombinant	¼ 1-(1 - r) ²
aB	Recombinant	¼ 1-(1 - r) ²
ab	Parental	¼ (1 - r) ²

For a cross between two homozygote individuals with the genes A and B in ***repulsion*** phase, with recombination frequency **r**, the gametic frequencies will be:

Gamete	AB	Ab	aB	ab
Frequency	½ r	½- ½ r	½- ½ r	½ r

Thus, the genotypic array will be:

Genotype	Frequency
AABB	$\frac{1}{4}\ r^2$
AABb	$\frac{1}{2}\ r - \frac{1}{2}\ r^2$
AAbb	$\frac{1}{4} - \frac{1}{2}\ r + \frac{1}{4}\ r^2$
AaBB	$\frac{1}{2}\ r - \frac{1}{2}\ r^2$
AaBb	$\frac{1}{2} - r + r^2$
Aabb	$\frac{1}{2}\ r - \frac{1}{2}\ r^2$
aaBB	$\frac{1}{4} - \frac{1}{2}\ r + \frac{1}{4}\ r^2$
aaBb	$\frac{1}{2}\ r - \frac{1}{2}\ r^2$
aabb	$\frac{1}{4}\ r^2$

And the phenotypic array can again be calculated:

Phenotype	Haplotype	Frequency
AB	Recombinant	$\frac{1}{4}\ (2 + r^2)$
Ab	Parental	$\frac{1}{4}\ (1 - r^2)$
aB	Parental	$\frac{1}{4}\ (1 - r^2)$
ab	Recombinant	$\frac{1}{4}\ r^2$

In the analysis described in Chapter 5, the pairs of genes were arranged as follows in the parents:

Genes	Phase
WELL – y	Repulsion
WELL – sit	Coupling
sit – y	Repulsion

The phenotypic ratios for the F_2 population of the cross P1 (*WELL*$^+$ *WELL*$^+$ *y y sit*$^+$ *sit*$^+$) x P2 (*WELL WELL y*$^+$ *y*$^+$ *sit sit*) were:

Phenotype	*WELL*$^+$	*WELL*	*sit*$^+$	*sit*	*y*$^+$	*y*
y$^+$	101	39				
y	40	0				
WELL$^+$			112	29		
WELL			33	6		
sit$^+$					116	29
sit					24	11
Total	141	39	145	35	140	40

The recombination rates for each pair of genes can be calculated adding up the expected frequencies for recombinant phenotypes in F_2:

WELL and *y* (repulsion):

$(1 + r^2)/2 = (101+0)/180 = 0.561$

$r = 0.349$

WELL and *sit* (coupling):

$[1 + (1 - r)^2]/2 = (112+6)/180 = 0.655$

$r = 0.443$

sit and *y* (repulsion):

$(1+ r^2)/2 = (116 + 11)/180 = 0.705$

$r = 0.641$

When r = 50% two loci are inherited independently and the distance between them in cM is infinite. This means that when two loci are inherited independently, one cannot be determine how many cM there is between the two loci. In the case of *sit* and Y, the resulting value is r = 0.641, considerably in excess of 0.5, which could be the result of negative interference between alleles.

4C - Chromosome *1* markers used to genotype WELL. "Pos." indicates genetic distance in centiMorgans from the top of chromosome *1*. "Marker type" refers to the type of marker, either SCAR (Sequence Characterized Amplified Region) or CAPS (Cleaved Amplified Polymorphic Sequence). "Enzyme" is the type of restriction enzyme used to reveal polymorphism in the CAPS markers, "Amplicon size" is the expected amplicon size after enzyme digestion in base pairs, for either the *S. pennellii* (*pen*) or *S. lycopersicum* (*lyc*) polymorphism, "Ann Temp" the annealing temperature in °C of the PCR primers, Fwd and Rev primer are the primer sequences.

Marker ID	Pos. (cM)	Marker	Enzyme	Amplicon size (bp) pen/lyc	Ann T°	Fwd primer	Rev primer
C2_At5g35360	21.3	SCAR	-	500/450	55	AACAGATCCGTGTCGCTATGGGAG	TTGAATGTCCTCTAAGCACAATATCCTC
TG236	23	CAPS	Dra I	650+350/ 1000	55	AGCCCAAGAAAACCACCCAAAGTG	GGGTCGAGAGATTACATAACGTTG
C2_At1g14310	30.5	CAPS	Alu I	200/100	55	TAGTGACGATACCTACTTTGAAGAAG	TCAGTATTGTGTCATAGGCTCCAGC
C2_At5g49480	31.6	CAPS	EcoRV	400/360	55	ACCAAATCTGCGGTCAGCATTCG	TTGAAGAAAACCTTGATAGGTAACACC
C2At4g15790	33.3	CAPS	Hinc II	1400/1050+	52	ATTGACTGAGTCAAGGCAAGAGCTGC	TAAGTTTCTTAAGCTGTTGGTAACATC
C2_At4g15530	42.6	CAPS	ScaI	650/700	55	TGTAAAGATGGTGGAGCCACAGC	ATCCACGGGCTACAACAGCTGC
C2_At5g13450	46.9	SCAR	-	550/500	55	AGGTTTGCTGAGTTAACCATGGC	TCTGTTCAATGTTCACCTTCTTCCC
C2_At3g08030	47.4	SCAR	-	750/800	55	AGGCTTCAATTTCTCAGACAATTCC	TTCAAAACCAGCATTTTTAACCAAG
U213330	49	CAPS	Hinf I	190/280	55	TCATCATGATCAAGCCTGATGGTGTC	AGAGAATCCTTTCTTCTCAAATCTGC
TG224	53	CAPS	Taq I	500+300/900+ 400	55	TCAACCTCCCACCTAGAAGGAAC	GAACCTTGCCACATACAAAATCATG
C2_At1g48050	58	CAPS	Taq I	750/920	55	TGAAAGAGGAAATAGGAGGATATGAAC	ATCGTGACTGGCGATATATTACGAG
TG59	62	CAPS	Bst N I	440/289	55	TCAGCGTTATGTTGCTCCAG	TGGAAGTGCTTCACGCTCTA
C2_At2g45620	69.2	CAPS	Dra I	850+350/ 1200	52	TATGGATCATGTGCCAATTCTTTTG	TCGAAGCAGCTTTGTGTTAACAACAG
C2_At2g45910	73	CAPS	ApoI	1100/650	55	TGACCCAGCCTTGAAGATTGGAGAAG	AGTGCAGGAATGGAGGAAGATGAGAGC
T1409	77	CAPS	SacI	500/250	55	ATGTGATACCTCCGCCGTTA	TTTCTTCCATTTCCCAACCA
C2_At2g38730	92.5	SCAR	-	500/550	55	AGCGGACCAAACACTAATGGATG	AGCCACATTCTCAATCTTCCTGAC

4D – Generation of recombinants using morphological markers

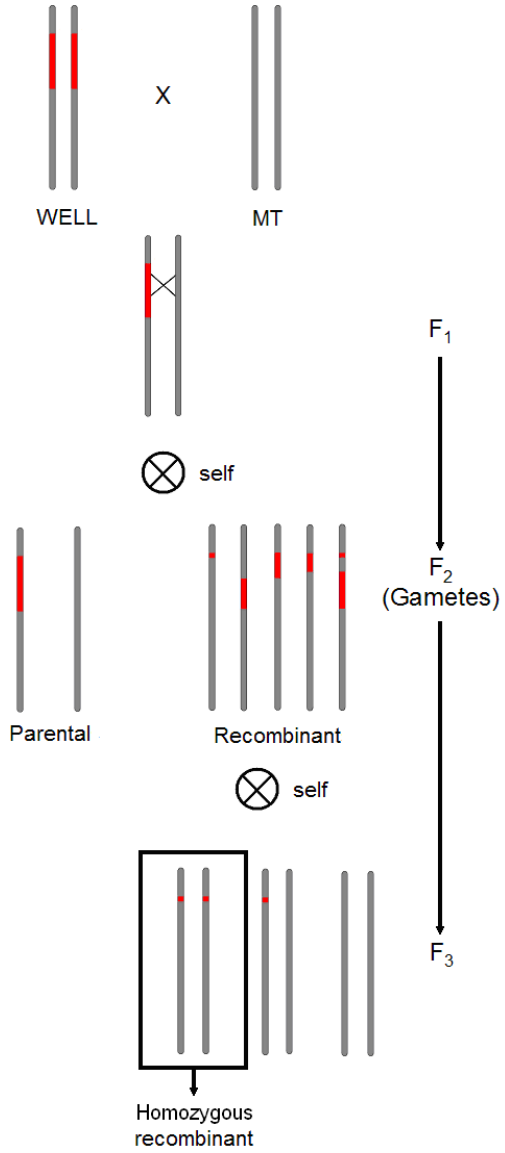

Figure 4D. Diagram showing the series of crosses made to obtain recombinant WELL plants using only morphological markers. The homozygous recombinant depicted in the bottom panel represents a tall plant (*WELL*$^{+}$-) with red fruit ($y^{+}y^{+}$). The *S. pennellii* genomic introgression is shown as a red bar, (bar sizes approximately to scale).

4E - F_2 phenotyping and genotyping

Each marker was scored for polymorphism between *S. pennellii* (pen), *S. lycopersicum* (lyc) and heterozygote (het). Only plants which had been previously phenotyped (see Chapter 4 for details) were genotyped in their entirety, *i.e.* using all the markers. Recombinants are represented in **boldface**. The plant ID numbers are not correlative because of plant mortality (numbering was not altered after removing sick/dead individuals). NB: The recombinant line #18 mentioned in Chapter 5 was obtained in a previous experiment and is not the same as individual #18 of this population.

Table 4E. Genotyping and phenotyping of a segregating F_2 population. Marker name (Mark), degree of wilting (Wilt) and plant height in mm (Heig). Dead or missing individuals are marked with asterisks (*).

Mark	49480	15530	13450	08030	48050	TG59	45910	Wilting	Height
cM	31.6	42.6	46.9	47.4	58	62	73		
1			pen				pen	0	170
2		het	het	het				1.5	170
3			pen	pen				0.5	180
4	**lyc**	**lyc**	**lyc**	**lyc**	**het**		**het**	3	145
5		het	het					1	190
6		het	het	het				2	170
7	*	*	*	*	*	*	*	*	*
8	lyc	lyc	lyc		lyc		lyc	3	110
9	lyc	lyc	lyc		lyc		lyc	2	90
10	het	het	het	het	het	het	het	2	200
11			pen					0	
12	het	het	het	het				2	210

13	*	*	*	*	*	*	*	*	*
14		het	het					1	140
15		het	het	het				2	200
16	*	*	*	*	*	*	*	*	*
17		het	het				het	1.5	110
18		het	het	het				1.5	140
19		lyc	lyc		lyc		lyc	1	130
20	*	*	*	*	*	*	*	*	*
21		het	het	het				2	230
22			het	het				0.5	150
23		het	het	het				1	170
24			pen	pen				0	165
25	*	*	*	*	*	*	*	*	*
26		het	het	het				2	190
27			pen					0	230
28			pen	pen					
29			pen	pen				0	155
30	*	*	*	*	*	*	*	*	*
31	*	*	*	*	*	*	*	*	*
32		het	het				het	2.5	180
33		het	het	het				0.5	165
34		het	het				het	1	160
35	lyc	lyc	lyc		lyc		lyc	0	95
36	*	*	*	*	*	*	*	*	*
37			pen	pen				0	175
38			pen	pen				0	210
39		het	het				het	0	140
40	*	*	*	*	*	*	*	*	*
41	**het**	**het**	**pen**	**pen**	**pen**	**pen**	**pen**	0	150
42			het	het				1	230

43	**lyc**	**lyc**	**lyc**	**lyc**	**het**	**het**	het	1	130
44			pen				pen	0	210
45	**het**	**lyc**	**lyc**	**lyc**	**lyc**	**lyc**	**lyc**	2	210
46		het	het	het				2	210
47		het	het	het				1	185
48	**lyc**	**lyc**	**lyc**	**lyc**	**lyc**	**lyc**	**het**	2	130
49	het	het	het	het			het	1.5	205
50	**het**	**het**	**het**	**het**	**het**	**lyc**	**lyc**	0	160
51	*	*	*	*	*	*	*	*	*
52		het	het	het				0	150
53		het	het					0	155
54	**lyc**	**lyc**	**lyc**	**lyc**		**lyc**	het	0	75
55			pen					0	265
56	*	*	*	*	*	*	*	*	*
57	*	*	*	*	*	*	*	*	*
58		het	het	het				1	100
59		het	het	het				0	170
60		het	het	het				0.5	150
61		het	het	het				1	145
62	*	*	*	*	*	*	*	*	*
63	**het**	**het**	**het**	**het**	**het**	**lyc**	lyc		
64	lyc	lyc	lyc	lyc	lyc	lyc	lyc	1	115
65			lyc					0	150
66	*	*	*	*	*	*	*	*	*
67			pen	pen				0	180
68		lyc	lyc					1.5	85
69			pen					0	220
70		het	het	het				1	180
71		het	het	het				1	160
72		het	het	het				1	190

73		het	het	het				1.5	175
74		het	het					1	150
75		lyc	lyc					2.5	110
76		pen	pen				pen	1.5	210
77	*	*	*	*	*	*	*	*	*
78	*	*	*	*	*	*	*	*	*
79	lyc	lyc	lyc		lyc		lyc	2.5	130
80		het	het	het			het	2.5	125
81			het	het			het	2.5	160
82		pen	pen					0	120
83	*	*	*	*	*	*	*	*	*
84		het	het				het	1	140
85		het	het				het	1	155
86		het	het					1	160
87		het	het				het	2.5	210
88			pen					0	160
89		het	het				het	0	160
90			pen					0	170
91	*	*	*	*	*	*	*	*	*
92		het	het					2	170
93	pen	pen	pen					0.5	190
94		het	het					0	230
95	*	*	*	*	*	*	*	*	*
96		het	het					1.5	160
97	lyc	lyc	lyc		lyc		lyc	2	97
98		het	het				het	0.5	130
99		lyc	lyc					2.5	80
100			pen					0.5	220
101								15	200
102	*	*	*	*	*	*	*	*	*

103	lyc	lyc	lyc		lyc		lyc	3	90
104		het	het					1	150
105	pen	pen					pen	0.5	210
106			pen						
107			het					1	180
108								0.5	220
109	pen	pen	pen				pen	0	160
110		het	het						
111			pen					0	185
112			pen					1	180
113	*	*	*	*	*	*	*	*	*
114		het	het					2	180
115	lyc	lyc	lyc		lyc		lyc	0.5	100
116			pen					0.5	240
117		het	het					1.5	210
118		het	het					2	180
119			pen					0	170
120		het	het					1	170
121		het	het					2	185
122			pen					0.5	180
123			pen					1	200
124			pen						
125		het	het					1.5	190
126	*	*	*	*	*	*	*	*	*
127			pen					0.5	210
128			pen					0	185
129	lyc	lyc	lyc		lyc		lyc	2	95
130	**lyc**	**lyc**	**lyc**	**lyc**	**het**	**het**	lyc	1	145
131		het	het						
132			het					2	190

133			het					1	190
134	lyc	lyc	lyc		lyc	lyc	het	3	110
135			het					0	150
136			het					1	160
137		lyc	lyc		lyc		lyc	2	100
138			pen					1	210
139		lyc	lyc		lyc		lyc	1	80
140	het	het	het	het			het	1.5	75
141			pen					0	190
142			het					1	165
143								0	200
144		lyc	lyc		lyc		lyc	1.5	65
145			pen					1	180
146	*	*	*	*	*	*	*	*	*
147	*	*	*	*	*	*	*	*	*
148	het	het	het	het	het		het	1	190
149			het					1.5	200
150			het					1	160
151			pen					0	210
152			het					0.5	175
153			het					1	165
154			het					0.5	200
155			het					0.5	180
156			pen					0	150
157			pen					1	240
158			het					1	190
159			het					1	240
160	*	*	*	*	*	*	*	*	*
161			het					2.5	240
162								2	230

163	het	1.5	155
164	pen	0	190

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