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Review

Improving Plant Water Use Efficiency through Molecular Genetics

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Abstract: Improving crop performance under water-limiting conditions is essential for achieving environmentally sustainable food production. This requires significant progress in both the identification and characterization of key genetic and physiological processes involved in water uptake and loss. Plants regulate water uptake and loss through both developmental and environmental responses. These responses include: root morphology and architecture, cuticle development, stomatal development, and guard cell movements in response to the environment. Genes controlling root traits and stomatal development and guard cell movements strongly impact water use efficiency (WUE), and represent the best targets for molecular breeding programs. This article provides an overview of the complex networks of genes involved in water uptake and loss. These traits represent novel opportunities and strategies for genetic improvement of WUE and drought tolerance in crops.

Keywords: WUE; water use efficiency; drought tolerance; stomatal; guard cell; cuticle; water loss; water uptake

1. Introduction

With a global excess of 663 million people worldwide lacking access to safe water, new sustainable water resource management strategies are necessary [1]. The increasing world population and the global water shortage represent the major challenges facing agriculture today [2], thus agriculture must provide for the continued increasing demand of food while addressing water-use practices [3,4]. Existing fresh water sources face competition between human consumption and agriculture compounded by an exponential increase in world population and a 70% increase in food requirements [3,5]. Global agriculture represents the major consumer of worldwide fresh water, about four fifths of the total [6]. Addressing demands for increased productivity and quality within the limits of current technology will not be easy and the challenge will be exacerbated by factors such as competition for fresh water by urbanization, increased land and water use from non-food and biofuel-crops, and especially by climate change [6,7].

Water-use efficiency (WUE) is broadly defined as the ratio of water used by the plant for metabolism to the water lost through transpiration [6]. The early consequences of global climate change have started to affect agriculture due to a continuous increase in CO_2 and greenhouse gasses in the atmosphere. Global temperature is predicted to increase by 2.5–4.3 °C by the end of the

century [8]. These environmental changes will increase plant photorespiration, nighttime respiration and consequently reduce plant growth [9]. Higher temperatures and heat waves are becoming more frequent, compounded by increasing episodes of drought, flood and land degradation [10]. Drought has been identified as the most detrimental environmental stress affecting agriculture worldwide and responsible for the greatest loss of yield of field crops [11]. In fact, previous studies estimated that drought stress results in an average yield loss of at least 50% in rice [12], chickpea [13], and of 18%–32% in potato [14]. This particularly affects developing countries in tropical and sub-tropical regions where climate change is predicted to have the greatest impact. In the coming decades, the occurrence of drought stress will become endemic worldwide due to climate change, making this crisis pivotal also for industrial nations [2]. Drought can afflict plants at any growth stage and can affect the productivity depending on the degree, intensity, and duration. Water represents the main requirement for crop production and is often a limiting factor for growth and yield. Over 90% of the water used by terrestrial plants is not utilized by biochemical processes, but is lost through transpiration [15]. Approaches to improving food production that combine improved water use efficiency will be critical in creating sustainable solutions. Most high-yielding crop varieties have been bred for maximized yield in conditions of ample water availability and do not possess suitable traits for yield and productivity under drought or water-limited conditions. Models linking weather and climate trends to the 1980–2008 yields of maize and wheat indicated that the production declined by 3.8% and 5.5%, respectively [16]. Specific reductions in yield in China and in developing countries [16] are expected for other important crops such as soybean or rice. These models and forecasts highlight the need to develop new varieties with increased drought tolerance and WUE combined with other high-yield traits [17]. Tolerance to drought in cereals is a complex phenotype controlled by several major QTLs (Quantitative trait locus) and small effect genes, many of which have yet to be functionally characterized [18]. Plants utilize a number of different mechanisms to survive conditions of water scarcity. Identification and combination of traits that contribute to tolerance and WUE represent a significant challenge if they are to be integrated in a manner that does not affect yield or productivity. Therefore, a full physiological and molecular dissection of drought responses is the first step in order to understand the complex mechanisms that control drought tolerance and WUE for use as traits in molecular breeding programs [2].

WUE represents a performance parameter that can reduce competition for fresh water, increase productivity and survival during periods of drought stress, and contribute to sustainability. However, this parameter is a complex composite governed by many genes and environmental responses. WUE remains hard to define exactly, and is often defined differently in the literature based on contrasting emphasis on contributing factors and productivity. De Witt [19] sets the original equation to explain the relationship between plant production and water use, where Biomass = mT/E_0. In this equation, *m* is equal to crop constant (a constant based on photosynthesis that differs between C3 and C4), T is equal to crop transpiration, and E_0 is equal to free water evaporation. Other methods put emphasis on water inputs and harvest index. Many studies in the literature have used yield defined as Y = WU (Water use) × WUE × HI (Harvest index) with WUE equal to biomass/water use ratio (WUE = B/WU) [20,21]. More recently, Keenan et al. [22] suggested another relationship, defining WUE as the ratio between water loss and carbon gain. Recently, Flexas [23] defined the relationship between photosynthetic capacity and WUE as WUEi = A_n/gs. In this equation, A_n corresponds to net CO_2 assimilation and *gs* corresponds to stomatal conductance. This latter aspect represents a central point in defining WUE for improvement because usually a higher WUE value results in lower stomatal conductance, but this is often linked to a decrease of photosynthetic capacity, reducing plant growth and productivity [24]. Therefore, the challenge to improving WUE must involve balancing reducing transpirational water loss with CO_2 uptake efficiency. WUE can be improved using different approaches that include genetic improvement and agricultural practices, selecting several associated traits including: photoprotective processes, antioxidant systems, regulation of acquaporins, signaling and transduction pathways as ABA (Abscisic acid) and regulation of photosynthesis [2,25].

In recent years, new crop varieties with improved drought tolerance and/or WUE have been developed through conventional breeding approaches using molecular assisted selection [11]. Conventional breeding has used selection of the drought-resistant genotypes as major QTL donors for introgression into high-yielding/drought-susceptible varieties [26]. Landraces or wild-relatives are proving to be deep sources of biodiversity and novel traits that improve stress tolerance and WUE [27]. Novel QTLs have been identified that represent traits involved in improving water-soluble carbohydrates, the carbon isotope ratio, osmotic potential, chlorophyll content, relative water content, leaf osmotic potential, osmotic adjustment, chlorophyll and chlorophyll fluorescence and others [2]. Particularly for WUE improvement, interesting candidates are to be found among stomata sensitivity and the ABA signaling pathway. Manipulation of these key processes are promising approaches to obtain drought tolerance and encouraging results have already been achieved in crops such as tomato or sunflower [27]. Plant microRNAs (miRNAs) may also play a role in manipulating expression of key genes related to drought tolerance and WUE. These miRNAs are approximately 20–24 nt-long small regulatory non-coding RNAs, abundant in higher organisms. Recent studies have determined that plants utilize miRNAs as critical post-transcriptional regulators of gene expression in response to different abiotic stresses. These small RNAs regulate gene expression via translational inhibition. In recent years, research has mainly aimed to identify plant miRNAs, responsive to single or multiple environmental factors, profiling their expression patterns and determining their roles in stress responses and tolerance [28]. In fact, many altered expressions of miRNAs have been reported in several plant species subjected to abiotic stress conditions such as drought, salinity, extreme temperatures and heavy metals. Although miRNAs and their role in controlling WUE is still nascent, miRNAs could be used as potential targets for genetic manipulation to engineer abiotic stress tolerance in crop plants. This review will analyze in depth the plant physiology of water management, analyzing the strategies of water loss reduction through stomata density, stomata opening/closure, and ABA signaling. We have focused on water uptake efficiency and genes that have been found to be involved in WUE and drought tolerance (Table 1).

2. Physiology of Water Use and Loss

Water plays a crucial role in the life of a plant. It is the most abundant constituent of most organisms. Water typically accounts for more than 70 percent, by weight, of non-woody plant tissues. The water content of plants is in a continual state of flux. The constant flow of water through plants is a matter of considerable significance to their growth and survival. The uptake of water by cells generates a pressure known as turgor. Photosynthesis requires that plants draw carbon dioxide from the atmosphere, while, at the same time, it exposes them to water loss through the stomata. To prevent leaf desiccation, water must be absorbed by the roots and transported through the rest of the plant. Balancing the uptake, transport, and loss of water represents an important challenge for plants. The thermal properties of water contribute to temperature regulation, helping to ensure that plants do not cool down or heat up too rapidly. Water has excellent solvent properties. For many of the biochemical reactions that occur in water, water is itself either a reagent or a product.

The practice of crop irrigation reflects the fact that water is a key limiting factor in agricultural productivity. Water availability likewise limits the productivity of natural ecosystems. Plants use water in vast amounts, but only a small part of that remains in the plant. Up to 97% of water taken up by plants is lost to the atmosphere, where the remaining 2% is used for volume increase or cell expansion, and 1% goes to metabolic processes, predominantly photosynthesis. Water loss to the atmosphere appears to be an inevitable consequence of carrying out photosynthesis. The uptake of CO_2 is coupled to the loss of water. Because the driving gradient for water loss from leaves is much larger than that for CO_2 uptake, as many as 400 water molecules are lost for every CO_2 molecule gained.

Table 1. Genes regulating water uptake and loss.

Gene	Gene Name	Protein Function	Species	Mutant Type	Expected Effect on WUE	Ref.
		Uptake through roots				
NAC9	NAC DOMAIN CONTAINING PROTEIN 9	NAM-ATAF-CUC	*O. sativa*	overexpression	increase	[49]
MYB96	MYB DOMAIN PROTEIN 96	R2R3-type MYB TF	*A. thaliana*	overexpression	increase	[155]
AN3	ANGUSTIFOLIA3		*A. thaliana*	loss of function	increase	[47]
		Stomatal density				
SDD1	STOMATAL DENSITY AND DISTRIBUTION 1	serine protease	*A. thaliana*	overexpression	increase	[57]
GTL1	GT2-like 1	GT2-like TF	*A. thaliana*	loss of function	increase	[53]
EPF1	EPIDERMAL PATTERNING FACTOR 1	cysteine-rich peptide	*A. thaliana*	loss of function	decrease	[61]
EPF2	EPIDERMAL PATTERNING FACTOR 2	cysteine-rich peptide	*A. thaliana*	loss of function	decrease	[61]
EPFL9	EPIDERMAL PATTERNING FACTOR-LIKE 9	cysteine-rich peptide/signalling pepetide	*A. thaliana*	overexpression	decrease	[156]
ERECTA	QRP1, QUANTITATIVE RESISTANCE TO PLECTOSPHAERELLA 1	LRR-like kinase	*A. thaliana*	loss of function	decrease	[157]
YODA	MAPKKK4, MAP KINASE KINASE KINASE 4	MAPKK kinase	*A. thaliana*	loss of function	decrease	[67]
AN3	ANGUSTIFOLIA3		*A. thaliana*	loss of function	increase	[47]
		Cuticle				
CER1	ECERIFERUM1	alkane biosynthesis	*C. sativus*	knockdown mutant	increase	[98]
SHN1	WAX INDUCER1/SHINE1	AP2/EREBP transcription factor	*S. lycopersicum*	overexpression	increase	[99]
CER9	ECERIFERUM9	cuticle biosynthesis	*A. thaliana*	loss of function	increase	[101]
		Stomata aperture and responses				
SLAC1	SLOW ANION CHANNEL-ASSOCIATED 1	S-type anion channel	*A. thaliana*	loss of function	decrease	[158]
SLAH3	SLAC1 HOMOLOGUE 3	S-type anion channel	*A. thaliana*	loss of function	decrease	[109]
ALMT12 or QUAC1	ALUMINIUM-ACTIVATED ANION CHANNEL 12 OR QUICK ANION CHANNEL 1	R-type anion channel	*A. thaliana*	loss of function	decrease	[112]
ABCB14	ABC TRANSPORTER B FAMILY MEMBER 14	ATP-binding cassette (ABC) transporter	*A. thaliana*	loss of function	increase	[113]
GORK	GUARD CELL OUTWARD RECTIFYING K+ CHANNEL	outward potassium channel	*A. thaliana*	loss of function	increase	[115]
KAT1	K+ CHANNEL ARABIDOPSIS THALIANA 1	Potassium channel protein	*A. thaliana*	dominant negative	increase	[159]

Table 1. *Cont.*

Gene	Gene Name	Protein Function	Species	Mutant Type	Expected Effect on WUE	Ref.
OST2 or AHA1	OPEN STOMATA 2 OR H(+)-ATPASE 1	proton ATPase	*A. thaliana*	dominant mutant	decrease	[119]
pyr1/pyl1/pyl2/pyl4/pyl5/pyl8	PYRABACTIN RESISTANCE/PYR1-LIKE	ABA receptor	*A. thaliana*	sextuple mutant	decrease	[160]
ABI1	ABA INSENSITIVE 1	protein phosphatase 2C	*A. thaliana*	loss of function	increase	[161]
HAB1	HOMOLOGY TO ABI1	protein phosphatase 2C	*A. thaliana*	loss of function	increase	[161]
OST1 or SnRK2.6	OPEN STOMATA 1 OR SNF1-RELATED PROTEIN KINASE 2.6	Ser/Thr kinase	*A. thaliana*	loss of function	decrease	[132]
HT1	HIGH TEMPERATURE 1	protein kinase	*A. thaliana*	dominant-negative	increase	[144]
NCED1	9-CIS-EPOXYCAROTENOID DEOXYGENASE	dioxygenase	*S. lycopersicum*	overexpression	increase	[162]

Notes: WUE (Water use efficiency); A. thaliana (Arabidopsis thaliana); O. sativa (Oryza sativa); C. sativus (Cucumis sativus); S. lycopersicum (Solanum lycopersicum).

2.1. Controlling Water Uptake through Roots Architecture

Plant roots play an essential role in the uptake of water and nutrients necessary for plant growth. They also serve as storage organs and anchor plants to the soil. The spatial distribution of all root parts in a particular growth environment is referred to as root system architecture and defines the available zone of water and nutrients. This plays an important role in abiotic stress tolerance, crop performance and yield [29]. Root architecture is dynamic and affected by the external environment that impacts the way in which a plant detects and responds to its surroundings [30,31]. Different root characteristics and morphologies enable plants to increase water uptake efficiency to adapt and to grow in different environments.

Recently, attention to the plant root system as a promising target for breeding crops to optimize growth in unfavorable environmental conditions has come to the forefront. Breeding for superior root systems may be decisive for selecting water efficient crops that can lead to dehydration avoidance via efficient uptake compatible with high yields [21,32,33]. Drought has a significant effect on development of the root system, with many plants preferentially increasing primary root elongation and suppressing lateral root branching in response to stress. Many plants adapted to drought, such as sorghum, have a naturally more vertically oriented root structure [34]. Many studies have identified different genetic components that affect root characteristics; some of these have the potential to improve the water use efficiency to limit crop loss due to adverse environmental conditions [35]. Rooting depth is one of the most commonly evaluated traits because crops with deeper roots have better access to water and nutrients [36]. Although rooting depth is strongly influenced by the soil's physical and chemical properties [37], recent studies have uncovered other factors that affect rooting depth, which could be exploited for crop breeding programs.

Zhan et al. [38] reported that maize recombinant inbred lines with few, but long, lateral roots had substantially deeper rooting, greater leaf relative water content, greater stomatal conductance, and 50% greater shoot biomass than lines with numerous short roots. Under water stress, these recombinant lines had 144% greater yield than controls. Another factor that influences rooting depth is gravitropism. The gravitropic response of roots was observed in a recent study showing that the DEEPER ROOTING 1 (DRO1) quantitative trait loci, which results in steeper root angles and more robust seedling gravitropic responses, leads to rice plants that are more tolerant to drought due to their deeper root system [39].

Root hairs are single cell protrusions that emerge from root epidermal cells. Because root hairs represent a large percentage of the total root surface area, they account for almost 50% of water absorption by the plant. The importance of root hairs for water uptake was shown by comparing wild-type Arabidopsis plants with hairless root mutants. Due to the reduced ability of the root hairless mutants to absorb water, they are more sensitive to drought, salinity and heat stress [40]. In barley, root hairs improved root penetration into hard, compact soils, a crucial feature for plant establishment [41]. A recent study in common bean showed that the benefits of root hairs for crop productivity can be improved in cultivars with superficial basal roots. The combination of long root hairs and shallow basal roots results in a synergistic effect on phosphorus acquisition that translates to a 300% increase in biomass in cultivars with both traits [42]. Several components of the molecular mechanisms that control root hair growth have been elucidated [43]. Modifying the expression of some of the genes that control root hair development has resulted in plants with longer and highly branched root hairs [44,45].

The formation of root branches or lateral roots is an important determinant of overall root system architecture. Lateral roots add to the total root biomass, total root length and root surface area. As such, it has been assumed that increased root branching density is associated with greater nutrient and water uptake [30,46]. However, like many other root traits, the ideal lateral root density is the result of both genetic traits and soil conditions.

In Arabidopsis, ANGUSTIFOLIA3 (AN3) acts as an important regulator of drought tolerance through the transcriptional repression of the MAPKK (Mitogen-activated protein kinase kinase) YODA (YDA). Plants lacking AN3 display increased WUE via improved root traits. The *an3* mutants show

enhanced primary root length and an increased number of lateral roots [47]. In rice, the NAC family of transcription factors was characterized with regard to root architecture [48]. One of these, OsNAC9, alters the root system, enhancing drought resistance and grain yield under field conditions [49]. The authors evaluated the overexpression of the TF OsNAC9 under the control of a constitutive or root-specific promoter in transgenic rice under both normal and drought conditions. In suboptimal water availability, grain yield was found to be improved in transgenic lines, which showed a reduced lateral root density.

Seo and Park [50] reported that MYB96 transcription factor, an Arabidopsis R2R3 type, regulates lateral root development under drought stress conditions via ABA-auxin signaling crosstalk, involving a subset of *GH3* genes encoding auxin-conjugating enzymes. The *MYB96*-overexpressing mutant, which is featured by having dwarfed growth and reduced lateral root formation, exhibits an improved drought tolerance and a significantly elevated expression of the *GH3* genes. Conversely, the *MYB96*-deficient knockout mutant had more lateral roots and was more susceptible to drought stress. These observations strongly support that *MYB96* is a molecular link that integrates ABA and auxin signals in modulating auxin homeostasis during lateral root development, especially under water deficit conditions.

In conclusion, above ground components of plants are well studied because they are accessible. Roots are less well studied because they are not readily visible and replicating the conditions in which they grow can prove difficult. Root traits, especially root length, density and depth, have long been seen as critical traits in order to improve water use efficiency and crop adaptation in non-optimal environmental conditions. The size and activity of the root system determines the rate at which the shoot system can produce photosynthates. It is evident that improving yield under stress conditions will require a whole plant approach.

2.2. Controlling Water Loss through Stomatal Density

The gas exchange between plants and atmosphere occurs mainly through stomata, the small pores on leaf and stem epidermis bordered by two guard cells. In response to different environmental factors, such as temperature, humidity, light, atmospheric carbon dioxide and drought, plants regulate the number of stomata in developing leaves. With this specific adaptation, plants control the water relations, minimizing water loss through transpiration and optimizing photosynthetic CO_2 fixation, and therefore, increasing WUE [51–53]. Stomatal number is determined by regulation of guard cell differentiation. Meristemoids, the small stomatal precursor cells, may undergo asymmetric divisions or convert into a guard mother cell (GMC). Subsequent symmetric division of GMC produces the two guard cells [54,55]. Those processes are regulated by several genes. Most of them are involved in fundamental development process and their manipulation leads to severe morphological effects. However, studies reported below identified genes whose manipulation causes change in stomata density without negative effects on growth and development.

A key determinant of stomatal development is a subtilisin-like serine protease, SDD1 (STOMATAL DENSITY AND DISTRIBUTION). This gene negatively regulates the differentiation of meristemoids and guard mother cells [56]. The modulation of *SDD1* expression decreases stomatal density, reduces transpiration and improves drought tolerance without reducing leaf area and/or plant size [57]. The expression of *SDD1* is directly repressed by the trihelix transcription factor GT2-like 1 (GTL1), a member of the GT-2 family. GTL1 regulates SDD1 through the specific binding to the GT3 box in the promoter area of *SDD1* [53]. Thus, in plants lacking *GTL1* expression, the negative regulation of stomatal development is potentiated. Loss of function mutants, in fact, display a reduction in stomata number and transpiration without changes in biomass accumulation, resulting in an increase in WUE [53,58]. In addition, members of EPF (EPIDERMAL PATTERNING FACTOR) and EPFL (EPIDERMAL PATTERNING FACTOR-LIKE) families regulate the stomatal density in developing Arabidopsis leaves. EPF/EPFLs encode small cysteine-rich secretory peptides, some of which are involved in different stages of stomatal development. *EPF1* is mainly expressed in guard cells of

stomata and stomatal precursor cells [59]. *EPF2* is expressed at earlier stages in meristemoids and guard mother cells [60,61]. Plants overexpressing *EPF1* or *EPF2* show severe reduction of stomata number without significantly changing the photosynthetic capacity, increasing WUE. Conversely, *epf1* and *epf2* mutants showed decreased WUE due to an increase in stomatal conductance via increased stomatal density [61]. EPF-Like9, also known as Stomagen, promotes stomatal differentiation. The overexpression of *EPFL9* increases the number of stomata, the opposite seen in *EPF1/EPF2* overexpression. This result indicates that EPFL9 positively regulates stomatal development and may compete for the same receptor of EPFs [62]. The secreted peptides, in fact, are recognized by cell surface receptors. It has been reported that the ERECTA family is the primary receptor for EPFs and that its activity is modulated by the LRR (Leucine-rich repeat) receptor-like protein, TMM (TOO MANY MOUTHS) [63]. *TMM* was the first gene identified to regulate stomata development [64]. The abolished expression of *TMM* increases stomatal number and leads to their clustering on leaf epidermis, especially on cotyledons. Conversely, *tmm* mutants show an absence of stomata on stems. This organ-specific pattern suggests that TMM has both positive and negative effects in stomatal development depending on where it is expressed. This might be related to the dissimilar availability of ligands in different organs [65,66].

YODA (YDA), an MAPKK kinase which functions downstream of TMM, acts as a regulator in guard cell fate determination [67]. Loss of function mutants showed increased stomatal numbers, while *YODA* overexpression produces plants completely lacking guard cells [47,67,68]. YDA is required to maintain the meristemoid equilibrium between proliferation and differentiation into GMCs. Therefore, *YDA* is downregulated to permit cells to enter the stomatal lineage. ANGUSTIFOLIA3 (AN3) acts as a main regulator of drought tolerance and WUE through the transcriptional repression of YDA. Arabidopsis plants lacking AN3 activity exhibit enhanced WUE through lower stomatal density and transpiration without a reduction in biomass accumulation [47].

In rice, Ishimaru et al. [69] studied the genetic relation between adaxial and abaxial stomatal density with quantitative trait loci (QTL) analysis on a population of backcrossed inbred lines of *japonica* variety, *Nipponbare* and *indica* variety, *Kasalath*. On chromosome 3, the QTL for adaxial density overlapped with the QTL for abaxial density, suggesting that the same locus may pleiotropically control stomatal density on both surfaces of the leaf. Laza at al. [70] detected four QTLs controlling the size of stomata and 10 QTLs controlling stomatal density across growth stages and leaf surfaces, using 101 recombinant inbred lines (RILs) derived from a cross between the *japonica* IR69093-41-3-2-2 and the *indica* IR72. The contributions of the QTLs ranged from 9.7% to 14.3% for stomatal size and 9.3% to 15.2% for stomatal density. However, the mechanisms that determine the observed phenotype are not completely known and detailed studies are needed to elucidate the function of the QTLs involved [11,70].

2.3. Controlling Water Loss through the Cuticle

In plants, water loss can be reduced by increased wax deposition in the cuticle, a reduced number of stomata per unit area, the presence of trichomes, reduction in leaf size and by the disposition of leaves with respect to the incident radiation [71]. Moreover, water loss is reduced by the nocturnal CO_2 uptake with an efficient synthesis of sugars and osmolytes, which allow water retention in CAM (Crassulacean acid metabolism) plants [72]. The presence of a prominent epicuticular wax layer increases WUE by reducing cuticular transpiration and increasing leaf boundary layer effects, and it decreases leaf and canopy temperatures by the reduction of net radiation reflecting solar radiation [73].

The surface of most plant tissues is covered with a thin cuticle layer consisting of a cutin polyester membrane impregnated and overlaid with waxes that supply the last barrier over essentially all aerial plant organs [74]. Cuticle structure, thickness, wax amount and composition are variable among plant species from different habitats, which may result in different cuticular transpiration [75,76]. The cuticle is synthesized by the epidermal cells and it preserves plants from non-stomatal water loss, dust deposits, pollen and air pollutants as well as biotic and abiotic stresses such as UV radiation

damage and bacterial and fungal pathogens [75,77–79]. Increased levels of cuticular waxes have been associated with improved drought tolerance in oat [80], sorghum [81] and rice [82]. A mutant of wild barley, *eibi1*, with a very thin cutin layer, was hypersensitive to drought [83]. Breeding for improved WUE, tolerance and yield under water deficit conditions led to increased amounts of cuticle waxes, further confirming the link between drought tolerance and cuticle properties [84]. Thus, the increased biosynthesis of cuticle waxes seems to be an established plant response to dry conditions.

Many sources of evidence suggest that both waxes and cutin are important in maintaining plant water status. This is made evident by studies using mutants defective in the composition of waxes but not cutin, such as the tomato *eceriferum6* (*cer6*) and positional sterile mutants [85,86], and mutants conferring alterations in both waxes and cutin monomers, such as *lacs1*, *lacs2*, *bodyguard*, *glossyhead1* [87–89]. Many genes coding for enzymes involved in the biosynthesis of cuticle components have been isolated and characterized [90–92].

Transcription factors (TFs) are involved in the regulation of biosynthesis and accumulation of cuticle components. Most of these belong to one of three different families: ethylene responsive factors (ERFs), myeloblastosis family (MYB) TFs and homeodomain-leucine zipper class IV (HD-Zip IV) factors [90,93–97]. Overexpression of these TFs leads to changes in cuticle accumulation and/or composition, often improving WUE and increasing stress tolerance. In many cases, overexpression of these TFs negatively affects plant growth and yield [93,97]. Recently, Wang et al. [98] isolated a *CER1* homolog *CsCER1*, a gene involved in alkane biosynthesis, in cucumber. They showed that abnormal expression of *CsCER1* in transgenic cucumber plants had strong effects on very-long-chain (VLC) alkane biosynthesis, cuticle permeability and drought resistance.

Aharoni and colleagues [93] identified an *Arabidopsis thaliana* mutant *shine* (*shn*) that displayed a brilliant, shiny green leaf surface with increased cuticular wax compared with the leaves of wild-type plants. The gene responsible for the phenotype encodes one member of a clade of three proteins belonging to the family of AP2/EREBP transcription factors. Overexpression of all three SHN clade genes conferred a phenotype similar to that of the original *shn* mutant. Moreover, SHN gene overexpression altered leaf and petal epidermal cell structure, trichome number, branching as well as the stomatal index and displayed significant drought tolerance and recovery. Expression analysis provides evidence for the role of the SHN clade in plant protective layers, such as those formed during abscission, dehiscence, wounding and the cuticle. The authors propose that these diverse functions are mediated by regulating metabolism of lipid and/or cell wall components.

The tomato ortholog of the transcription factor SlSHN1 was also isolated and the expression analysis indicated that it is induced in response to drought conditions [99]. Overexpression of SlSHN1 in tomato produced plants that showed mild growth retardation with shiny and dark green leaves. Expression analysis of these transgenic lines indicated that several wax-related synthesis genes were induced. Transgenic tomato plants showed higher drought tolerance compared to wild type plants; this was reflected in delayed wilting, improved water status and reduced water loss.

Zhou and colleagues [100] carried out a functional analysis of OsGL1-6 in rice. OsGL1-6 is homologous to CER1 in Arabidopsis and *Wda1* in rice, widely expressed in vegetative and reproductive organs, and especially highly expressed in leaf epidermal cells and vascular bundles. A phenotypic characterization and drought sensitivity experiments on OsGL1-6 antisense-RNA transgenic plants showed that OsGL1-6 is involved in cuticular wax accumulation and drought resistance. The drought susceptibility was in agreement with their deficient cuticles and positively correlated with the reduced accumulation of the leaf cuticular wax, implying its role in drought stress resistance. Thus, genetic modification of OsGL1-6 may have great potential for improving the drought resistance of rice. Studies on mutation of the ECERIFERUM9 (CER9) gene in Arabidopsis showed a dramatic shift in the cuticular wax profile (especially on leaves) toward the very-long-chain free fatty acids tetracosanoic acid (C24) and hexacosanoic acid (C26) [101]. Compared with the wild type, *cer9* mutants exhibit elevated cuticle membrane thickness over epidermal cells and cuticular ledges with increased occlusion of the stomatal pore. CER9 is the first described cuticle biosynthesis gene whose deficiency improves

both plant response to water deficit and WUE, indicating that CER9 may encode an important new cuticle-associated drought tolerance determinant. These studies provide evidence that the CER9 protein is a negative regulator of cuticle lipid synthesis via its putative role as an E3 ubiquitin ligase, similar to Doa10 in yeast. Due to its novel impact on plant water status, elucidation of CER9's cellular function may reveal new molecular breeding and transgenic strategies to improve the drought tolerance and WUE of crop plants.

Studies of the numerous and diverse wax mutants now available should highlight the specific contribution of single wax compounds in plant/environment interactions as well as in the organization of waxes, together with cutin, in the highly structured cuticle. Clearly, a co-regulation of cutin and waxes is required for both environmental and developmental purposes of the cuticle.

In conclusion, water use efficiency is an indicator related to plant growth and is a function of the amount of water used to produce biomass. Furthermore, the close relationship between anatomical parameters (in our specific case cuticle, but also stomata and roots), water use efficiency, and tolerance to sub-optimal environmental conditions requires a clear understanding of the components involved and how they are regulated in response to the environment.

2.4. Controlling Water Loss through the Guard Cells Movement and Signaling Transduction

Opening and closure of the stomata is mediated by changes in the turgor pressure of the guard cells. The turgor pressure is controlled by movements of large quantities of ions and sugars in and out of the guard cells. When guard cells take up these solutes, the water potential inside the cells decreases, causing osmotic water flow into the guard cells. This leads to a turgor pressure increase causing swelling of the guard cells and the stomatal pores to open. In 1929, Stalfelt [102] recognized that there are two phases in stomatal opening: 'Spannungs-phase' and 'Motorphase'. In the first phase, the guard cells build up the turgor by accumulating osmotically active substances and increase in size [102]. This increase does not yet result in the opening of the stomatal pore because the changes in the osmotic pressure of guard cells are expected to be relatively small [103]. Only in the second phase do the guard cells reach a certain threshold turgor during which any further accumulation of osmoyltes results in swelling and bending of guard cells.

The ions taken up by guard cells are mainly potassium (K^+) and chloride (Cl^-) ions. In addition to these ions, guard cells take up sugars that also contribute to opening of the stomatal pores. In fact, several plasma-membrane-associated ion transporters are responsible for membrane polarization and depolarization events necessary to induce water exit/entrance in the guard cells that cause stomatal closure/opening, respectively. There are two types of anions channels: Rapid (R)-type channels activate within milliseconds [104] and Slow (S)-type channels where the activation occurs in seconds [105]. Slow anion channel-associated 1 (SLAC1) is an S-type anion channel that transports malate to the apoplast, causing membrane depolarization and stimulation of the efflux of K^+ ions, followed by osmosis of H_2O [106,107]. SLAC1 was identified in two independent mutant screens, and loss of it leads to plants with open stomata that display impaired responses to ozone, CO_2 and decreases in external humidity [108]. Another S-type anion channel is SLAC1 homologue 3 (SLAH3). Geiger and colleagues [109] reported that loss of both channels leads to stomata that completely lack responses to hormone ABA and they display similar properties except for their permeability to $NO3^-$, which is much higher for SLAH3 than SLAC1 [109].

R-type anion channels are encoded by genes belonging to the family of aluminum-activated malate transporters (ALMT) [110]. Despite the name, the guard cell localized ALMT12 channel is not activated by aluminum and was subsequently renamed the quick-anion-channel-1 (QUAC1), which carries mainly chloride and nitrate currents [111]. Loss of QUAC1/ALMT12 causes stomata to close more slowly in darkness and at high atmospheric CO_2 concentrations [112]. These data suggest that R-type and S-type channels act in concert releasing anions and depolarizing the guard cell plasma membrane during stomatal closure.

ABC transporter B family member 14 (AtABCB14) plays an opposing role to SLAC1. It transports malate into the guard cells, preventing stomatal closure [113]. K^+-efflux channels in *Arabidopsis* guard cells are encoded by GORK (Gated outwardly-rectifying K^+) [114]. Hosy and collaborators [115] show in their work that stomata of *gork1* loss of function close more slowly than the wildtype, and mutants display an increased water loss caused by defects in stomatal closure. The phenotype of the *gork/kup6/kup8* triple mutant, reported in the work of Osakabe and colleagues [116], exacerbated the phenotype of *gork1* plants. The triple mutant exhibited enhanced cell expansion, suggesting that these KUPs (K^+ uptake permeases) negatively regulate turgor-dependent growth. During water deficit stress, the triple mutant impaired ABA-mediated stomatal closing and had decreased survival from drought stress. KUP6 and 8 are members of the HAK (High-affinity K^+)/KUP transporter family, which were originally described as encoding high-affinity H^+/K^+ co-transporters [117]. Based on the phenotype of the *gork/kup6/kup8* triple mutant, KUP6 and 8 provide a K^+-efflux mechanism in the plasma membrane guard cells that acts in parallel with the GORK channel [116]. K^+-influx channels in *Arabidopsis* guard cells are encoded by a family of Shaker-like genes including KAT1 (K^+ channel *Arabidopsis thaliana* 1), KAT2 (K^+ channel *Arabidopsis thaliana* 2), and KT1 (K^+ transporter 1). They mediate potassium influx into guard cells leading to stomatal opening [118]. Another essential component of stomatal opening is AHA1/OST2 (*open stomata 2*), encoding a plasma membrane proton ATPase responsible for the plasma-membrane hyperpolarization, which initiates stomata opening. Mutants have a reduced ability to close their stomata in response to drought and are affected in stomatal responsiveness to ABA [119].

When water scarcity conditions are perceived, regulation of these effectors to induce stomatal closure is achieved through an increase in ABA concentration within guard cells. The first step for stomatal closure is the ABA-induced activation of S- and R-type ion channels in the plasma membrane [120]. The activation of these channels results in a depolarization and causes the extrusion of anions and K^+ from guard cells. In addition to the activation of anion channels, ABA also inhibits the activity of plasma membrane H^+-ATPases [121], which is likely to inhibit reopening of stomata.

ABA is perceived by the pyrabactin resistance (PYR)/PYL (PYR1-like)/regulatory component of ABA response (RCAR) family of intracellular ABA receptors [122,123]. Initially identified in Arabidopsis, the ABA receptors have since been described in many species such as tomato [124,125], beechnut [126], strawberry [127], rice [128], sweet orange [129], and soybean [130]. In the absence of ABA, protein phosphatase 2C (PP2Cs) binds to and inhibits snf1-related protein kinase 2 (SnRK2s). In response to environmental cues, ABA promotes the interaction of PYR/PYL/RCARs with PP2Cs, resulting in PP2C inhibition and SnRK2 activation. PP2Cs are a family of major negative regulators of ABA responses including ABA insensitive 1 (ABI1), ABA insensitive 2 (ABI2), homology to ABI1 (HAB1), and PP2CA [131]. In guard cells, regulation of membrane-located ion channels results in loss of turgor and closure of the stomata. In particular, the main target of PP2C inhibition is OST1 (open stomata 1, also known as SnRK2.6/SRK2E), a Ser/Thr kinase that constitutes a major hub for the regulation of immediate and transcriptional responses to ABA and is also involved in CO_2 responses. In addition to OST1, SnRK2.2 and 2.3 were found to be major targets of the type 2C protein phosphatases. Loss of these three genes results in Arabidopsis seedlings becoming virtually insensitive to ABA [132,133]. The activation of stomatal closure is the result of phosphorylated proteins located on the plasma membrane and activation of downstream effectors such as TFs (transcription factors). It was found that OST1 directly interacts and stimulates the S-type anion channel SLAC1, as well as the R-type channel ALMT12 [120]. This regulatory interaction seems to be a central molecular switch for triggering stomatal closure in response to ABA, CO_2, reduced air humidity, and darkness [108]. By contrast, OST1-induced phosphorylation results in the inactivation of inhibitors of stomatal closure. For example, phosphorylation at Thr306 results in reduction of the activity of KAT1 [134].

While various stimuli lead to stomatal closure, light predominantly provokes opening of these pores. It happens through distinct mechanisms in response to the different wavelengths. It is possible to distinguish two parallel signaling pathways: one is specifically sensitive to blue light, whereas the other is stimulated by photosynthetic active radiation (PAR) in general [135]. Blue light acts as

a signal, activating the H^+-ATPase pump via phosphorylation and promoting K^+ ions uptake [52]. Blue light receptors are phototropins; they are associated with the plasma membrane and function as blue light-activated protein kinases [136]. The activation of a H^+-ATPase pump is obtained via a signaling pathway involving type 1 protein phosphatases (PP1), whereas it inhibits S-type anion channels in guard cells [137,138]. This will promote hyperpolarization of the plasma membrane, leading to K^+ uptake into guard cells.

Red light acts both as signal and as energy source. The mechanisms through which the red irradiation controls the stomatal response have remained unclear for years. Red light drives photosynthesis in mesophyll and guard cell chloroplasts and leads to a reduction in intercellular CO_2 concentration that may cause stomatal opening [139,140]. The stomatal opening in response to red irradiation requires a high intensity of light. Previous studies showed that red light applied to the isolated epidermis induces H^+-ATPase activity in guard cell chloroplasts. This response is suppressed by DCMU (3(3,4-dichlorophenyl)-1-1-dimethylurea), an inhibitor of photosystem II in several species, indicating that the red light-induced stomatal opening is photosynthesis-dependent [141]. The lowering of intercellular CO_2 concentration due to red light deactivates anion channels in guard cells, increasing cell turgor that leads to stomatal opening [142]. Matrosova and collaborators [140] reported that the protein kinase HIGH LEAF TEMPERATURE 1 (HT1) is fundamental in mediating red light-induced stomatal opening. HT1 is a negative regulator of stomatal closing induced by CO_2. HT1 directly phosphorylates OST1 and inhibits OST1-induced activation of the S-type anion channel, SLAC1 [143]. The *ht1* mutant alleles do not show stomatal opening to low CO_2 concentration. Arabidopsis *ht1-2* mutant plants display a reduction in photosynthetic activity during increasing light conditions [140,144].

2.5. Plant Architecture and Branching Geometry

Plant architecture can reflect changes in WUE besides the effects described above, such as traits related to roots, stomata and cuticles. In fact, the three-dimensional organization of plants is mainly regulated by endogenous mechanisms, which may represent improvements in drought tolerance.

The axillary meristem represents the origin of lateral branches. A TB1 CYCLOIDEA PCF (TCP) type transcription factor, BRANCHED1 (*BRC1*), specifically regulates axillary meristem initiation causing suppression of branching. *Arabidopsis* plants lacking *BRC1* develop more rosette branches [145], whereas the overexpression of the *BRC1* ortholog in rice, *TB1*, leads to reduced number of tillers and improved culm thickness [146]. In *Solanum lycopersicum*, they identified two BRC1-like paralogues, SlBRC1a and SlBRC1b. These genes are expressed in arrested axillary buds and both are downregulated upon bud activation [147].

In addition, tiller angle in grain crops represents an important feature of plant architecture. *TILLER ANGLE CONTROL1* (*TAC1*) is a quantitative trait locus that controls the size and the tiller angle. Yu et al. [148] demonstrated that in rice the high expression of *TAC1* leads to a wider tiller angle, while the low expression results in a smaller angle [148]. Another gene that regulates tiller angle is PROG1, and it is a key domestication gene responsible for plant architecture [149]. Jin and collaborators founded that PROG1 contains a single highly conserved C2H2-type zinc-finger motif at its N-terminal region, suggesting that it serves as a transcription factor. In particular, they found that PROG1 is a nuclear transcription factor and that the activation domain is localized in the C terminus. In summary, these genes involved in plant architecture and branching geometry may play an important role for managing water and light resources, although their direct effects on plant response to drought stress have not been established.

3. Conclusions

Identification and integration of the numerous traits responsible for significant increases in WUE, which also prove effective in the field, represent a considerable challenge. The composite phenotype that results in a plant that uses less water while retaining its ability to assimilate carbon is composed

of many traits. These traits involve: root development, cuticle composition, stomatal development and responses, and intricate signaling transduction networks in response to environmental conditions. Integration of these traits may come at a cost in terms of growth and yield. Breeding programs that have selected for higher WUE have also resulted in selection for genes that reduce leaf area or promote early flowering [21]. QTL analysis of NILs (Near isogenic lines) of Arabidopsis have identified numerous QTLs involved with WUE, some co-localized with flowering-time QTLs involved with drought avoidance [150]. However, some of these genes have been shown to be independent from QTL analyses, and it is possible to select for higher WUE while leaving out flowering time QTLs [151].

When traits governing reproduction, vegetative growth, and WUE were examined, genotypes that demonstrated high WUE under water limiting conditions conversely had very low WUE under non-limiting conditions. These same genotypes also had the larger leaves and flowers [152]. In *Brassica rapa*, it has been suggested that it is possible to select for these traits without other maladaptive traits previously associated with WUE.

Optimal WUE and plant productivity may be a delicate balancing act. Imposition of drought on various *Brassica rapa* genotypes showed varying responses on allocation of biomass. Genotypes that moderately increased their root:shoot ratios in drought conditions showed higher photosynthesis levels and fruit yields than genotypes that allocated the most growth to root biomass [153]. Therefore, growth and expansion of the root system beyond the minimal requirements for photosynthesis can come at a cost to yield.

Greenhouse conditions often limit or eliminate the factor of competition in WUE experiments. Taking into account how plants perform in these conditions is essential for integrating potential WUE traits that may perform well in laboratory conditions, but may not prove suitable for the field. Competition between plants can affect WUE and overall plant performance. When multiple alleles of *MPK12* were grown in various combinations of drought and competition, it was found that alleles with high WUE did better in the absence of competition and lines with low WUE did better in competition [154]. All of the above examples indicate a distinct need to evaluate all of the genes discussed in this review in a context of ecophysiology with particular attention to the potential costs in regards to growth and yield. Of the numerous genes discussed in this review, only a handful have been evaluated for a WUE phenotype (Table 2). Furthermore, the potential interactions of these genes, either additive or subtractive, will need to be addressed in order to develop more WUE crops.

Molecular genetics represent an essential approach for identification and elucidation of the various traits that contribute to WUE. This review has addressed some of the better characterized genes that molecular genetics has identified that control water uptake and loss (Table 1). To fully utilize our knowledge of these genes to improve WUE, we need to utilize an integrated approach that implements functional characterization of promising QTLs, high-throughput phenotyping, field validation of traits, and stacking/pyramiding of these traits into WUE efficient and drought tolerant varieties for agriculture (Figure 1). This challenge represents one of the most complex tasks facing biotechnology today and will require both modern breeding and gene editing techniques to achieve. Regardless of the challenge, molecular genetics will be essential in identification and characterization of genes that play an important role in increasing WUE and drought tolerance.

Table 2. WUE (Water use efficiency) directly related genes.

Gene	WUE Related Trait	Species	WUE Measurment	Range of Variation	Tested Conditions	Ref.
AN3	Stomatal density	*A. Thaliana*	Leaf RWC	44% reduction in mutant	drought (19 days)	[47]
GTL1	Stomatal density	*A. Thaliana*	Integrated WUE, fresh shoot weight/water used (g/Kg)	2 times more in mutant	well-watered	[53]
EPF1	Stomatal density	*A. Thaliana*	WUE, assimilation rate/transpiration rate (mmol/mol)	3 times more in mutant	well-watered	[61]
ERECTA	Stomatal density	*A. Thaliana*	WUE, plant dry weight/water used (g/g)	1 time less in mutant	drought (16 days)	[157]
CER9	Wax accumulation	*A. Thaliana*	WUE, δ13C	50% reduction in mutant	well-watered	[101]

Notes: RWC (Relative water content); *A. thaliana* (*Arabidopsis thaliana*).

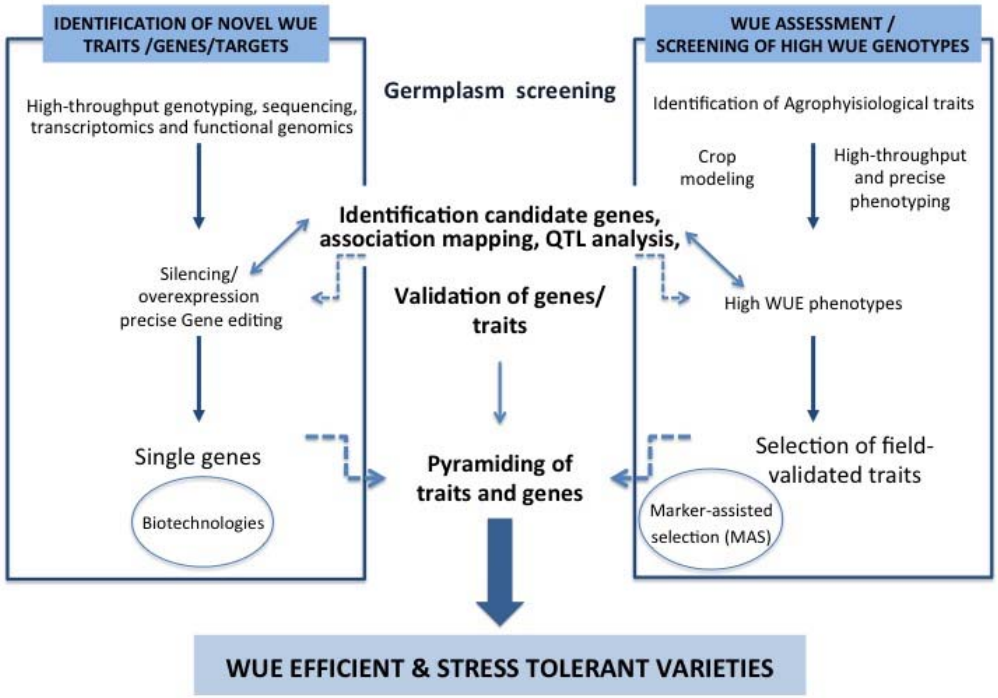

Figure 1. Strategy for Developing WUE (Water use efficiency) Efficient and stress tolerant crop varieties.

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