Chapter

Leaf Cuticular Wax, a Trait for Multiple Stress Resistance in Crop Plants

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Abstract

Cuticular waxes form the primary interface between a plant and its external environment. The most important function of this hydrophobic interface is regulation of non-stomatal water loss, gas exchange and conferring resistance to a wide range of biotic as well as abiotic stresses. The biosynthesis, transport and deposition of the cuticular waxes are tightly coordinated by complex molecular networks, which are also often regulated in response to various developmental, biotic as well as abiotic cues. Evidences from model as well as non-model systems suggest that targeted manipulation of the molecular regulators of wax biosynthetic pathways could enhance plant resistance to multiple stresses as well as enhance the post-harvest quality of produce. Under the current scenario of varying climatic conditions, where plants often encounter multiple stress conditions, cuticular waxes is an appropriate trait to be considered for crop improvement programs, as any attempt to improve cuticular traits would be advantageous to the crop to enhance its adaptability to diverse adverse conditions. This chapter briefs on the significance of cuticular waxes in plants, its biosynthesis, transport and deposition, its implication on plant resistance to adverse conditions, and the current options in targeted manipulation of wax-traits for breeding new crop types.

Keywords: cuticular waxes, wax biosynthesis, biotic stress, abiotic stress, stress resistance

1. Introduction

In the current era of increasing uncertainties in crop production, emerging constraints and risks demand technical and technological advances in the agricultural sector, and integrative approaches, such as Climate Smart Agriculture (CSA), to address the interlinked challenges of food security and climate change. While maintaining food security is a major challenge for future, the possible solution is to enhance crop productivity along with nutritional security. However, this stance is remarkably limited by the different abiotic as well as biotic environments, where the crops grow and develop.

Drought, excess water (flooding), extremes of temperatures (cold, chilling, frost, and heat), salinity, high and/or low light, mineral deficiency, and toxicity are the common abiotic stresses for crop production. These stresses alter plant metabolism, growth, development, and in extreme cases cause the cessation of

vegetative and reproductive growth. Some of the abiotic stresses such as drought, high temperature and salinity can influence the occurrence and spread of biotic agents like pathogens, insects, and weeds [1]. In crops like tomato, cucurbits and rice, temperature is one of the most important deciding factors for the occurrence of bacterial diseases [2]. Temperature can also alter the incidence of vector-borne diseases by modifying spread of vectors.

But, in their natural environment, plants face combination of stresses, especially under the changing climate scenario. The effect of stresses would be more pronounced under combined (biotic and abiotic) stresses [3], while simultaneous occurrence of abiotic and biotic stresses are more destructive to crop production [4]. Hence, there exists a need now, to look for common traits that can contribute for plant adaptation to such multifarious stressful conditions and sustain crop productivity as well. In this scenario, it is desirable to have a single trait that can confer tolerance to multiple (abiotic and biotic) stresses. Cuticular waxes, a major component of plant cuticle covering all the aerial parts of the plants, can be considered as an important trait for combined stress resistance.

2. Cuticular waxes: a component of plant cuticle

The cuticle is a unique structure developed by land plants during the course of their evolution from an aquatic to a terrestrial lifestyle [5]. The primary role of this lipophilic layer, comprising cutin and cuticular waxes, was to limit non-stomatal water loss by functioning as a physical barrier between the plant surface and its external environment [6]. Development of a cuticular barrier is one of the major adaptive mechanisms for survival and growth of plants under water limiting terrestrial conditions [7]. As the primary barrier between the aerial surface of plants and the external environment, the cuticle also protect the plants from mechanical rupture or injury, toxic gases and ultra violet radiation [8–10]. The cuticle also has notable roles associated with growth and developmental processes like preventing epidermal fusion by establishing normal organ boundaries [11], and phytohormone homeostasis [12]. The cuticle and its components are known to play essential roles as signaling molecules for pathogens and for the plants themselves [13]. Another important role is in fruits, where it influences quality, defense and post-harvest shelf life [14]. In fruits, water retention [15] firmness [16] and its responses to physical and biotic stresses are also influenced by the cuticle [17].

The cuticle is composed of a covalently linked scaffold of cutin and a mixture of soluble cuticular lipids (SCL), called as waxes [10, 18]. Structurally, cutin is made of covalently cross linked C16 or C18 oxygenated fatty acids and glycerol, forming the most abundant structural component of the cuticle [19]. The waxes within the cuticle function as an actual barrier against the diffusion of water or solutes [20, 21]. The waxes occur in two layers; forming two distinct physical layers called intra- and epi-cuticular waxes [22]. The former is dispersed within the cutin polymer while the epi-cuticular wax is deposited on the outer surface as crystals or films [22, 23]. This outermost layer can be physically stripped off the surfaces using aqueous glue [23, 24]. These waxes are composed of a variety of organic solvent-soluble lipids; consisting of very-long-chain fatty acids (VLCFA) and their derivatives. The major composition of VLFCAs are alkanes, wax esters, branched alkanes, primary alcohols, alkenes, secondary alcohols, aldehydes ketones, and unsaturated fatty alcohols, as well as cyclic compounds including terpenoids and metabolites such as sterols and flavonoids [19, 25–27]. Wax composition varies with crop species and differs in their functions and responses to biotic and abiotic environments [10].

As per recent studies, intra-cuticular waxes form the primary transpirational barrier and the contribution of epi-cuticular waxes as a transpirational barrier

depends on the species-specific cuticle composition [28]. In species like *Tetrastigma voinierianum*, *Oreopanax guatemalensis*, *Monstera deliciosa*, and *Schefflera elegantissima*, intra-cuticular wax pre-dominantly act as a transpirational barrier while in *Citrus aurantium*, *Euonymus japonica*, *Clusia flava*, and *Garcinia spicata*, both intra- as well as epi-cuticular waxes had equal contribution as transpirational barriers [28]. A study from *Prunus* suggests that intra-cuticular waxes of the cuticle form the actual transpirational barrier [29] and not epi-cuticular waxes [30].

3. Ecological significance of cuticular waxes

The cuticular waxes confer diverse surface properties to plant parts, which actually play the key role in controlling non-stomatal water loss and gas exchange, and protection from external environment. Leaf cuticular wax amount and crystal morphology regulated post-harvest water loss from leaves [31]. Epi-cuticular wax films give glossy appearance to leaves and fruits, while wax crystals (β-diketones) conferred dull, glaucous appearance to leaves and stems [10]. The thickness [5] composition and properties of the waxes vary with crop species and are found to be induced under diverse stressful conditions [32]. These differences reflect their functions and responses to biotic and abiotic environments [10]. Importance of cuticular wax accumulation in plant resistance to both biotic as well as abiotic stress conditions is now well documented [12, 33, 34].

3.1 Abiotic stresses

One of the most important roles of the waxes is to protect the plant surfaces from excessive solar and ultraviolet (UV) radiations. Cuticular waxes scatter UV-B radiation [35] and was demonstrated in apple [36]. As per studies from model systems as well as crops, increased cuticular wax biosynthesis improves drought stress resistance [37]. In rice, wheat, barley and sorghum, grain yield under water limiting conditions have positive correlation with wax content [38–41] . Hence, in crop plants, higher cuticular wax content is a promising trait for stress resistance as well as yield under water limiting conditions [27]. In mulberry, increasing wax load is useful to manage post-harvest water losses [42]. In barley, cuticular wax components act as a barrier to water loss and contribute to salt stress resistance [43]. Heat stress resistance is also positively correlated with wax accumulation in bahia grass [44]. Under heat stress, the wax load in sorghum was correlated with its ability to maintain the canopy temperature cool, resulting in reduced water loss [45]. Similarly, pea varieties with thicker wax load also exhibited lower canopy temperature, thereby limiting water loss and associated heat stress [46].

Cuticular waxes play an important role in preventing non-stomatal water loss during drought and high temperature stress, as well as enabling frost avoidance. Such climatic stressors can induce a heavier wax load and change the chemical composition of waxes by accumulating longer aliphatic compounds on plant tissues [47]. Drought increases stiffness and quality of the plant cuticle under climate change [48]. Similarly, the leaf cuticular surface is the first barrier blocking destructive ice penetration into the leaf cells in freezing avoidance mechanisms [49]. Using a hydrophobic film, Wisniewski et al. [50] showed the importance of the epi-cuticular hydrophobicity enabling avoidance of freezing in sensitive plants. The critical nature of the cuticular layer in frost avoidance of corn is also clearly demonstrated [51]. Freezing avoidance is the only mechanism of frost resistance in sensitive plants. In fact, the first demonstration of a transgenic organism in agriculture was the alteration of the cell wall protein secondary structure on ice nucleating bacteria, *Pseudomonas syringae* and *Erwinia herbicola*, which then prevented ice nucleation across the cuticle and avoided leaf damage [52, 53]. In future, injury due to frost stress will be more, not

less under global warming [54]. Hence, a better understanding of stress-induced wax modification among crop plants holds promise to cope with climate change.

3.2 Biotic stresses

The cuticle and its components act as signaling molecules to favor fungal growth and development, and infections in plants [55, 56]. Surface waxes act as cues to activate fungal developmental processes like appressorium formation, pre-penetration processes, etc., in crop plants like avocado, wheat, rice, maize and peanut [13, 57–59]. However, the hydrophobic nature of the cuticle also renders it a barrier for bacterial as well as fungal pathogens [60], a desirable trait for disease resistance. Waxes are known to protect lotus from pathogen infection [61]. It repulses pathogen spores and atmospheric pollutants like acid rain and ozone [32]. Another role of waxes is in plant-insect interaction; to attract or to serve as a deterrent [62]. It prevents insect attachment to plant surface oviposition and feeding [63, 64] and hence confer tolerance to insects in crop plants [65, 66].

4. Molecular biology of cuticular wax biosynthesis and deposition

Studies in *Arabidopsis* and subsequently, barley, rice and tomato systems have significantly contributed for the elucidation of the complex regulatory pathways underlying the biosynthesis, transport and deposition of wax components on plant surfaces [26, 27, 67]. Cuticular wax biosynthesis predominantly occurs in epidermal cells. The biosynthetic pathway initiates exclusively in the outer membranes of the plastids of epidermal cells where C16 and C18 fatty acids are synthesized, exported to the cytosol as acyl-CoAs and then elongated up to C34 at the endoplasmic reticulum (ER); through a series of enzymatic reactions [19, 26]. The synthesized components are subsequently transported through the apoplastic pathway and deposited on the cuticle. The key steps involved [32] are summarized here.

4.1 Synthesis of malonyl-CoA

The *de novo* fatty acid biosynthesis initiates with the synthesis of malonyl-CoA. It is initiated with the transfer of a bicarbonate derived CO_2 molecule to the biotin moiety of a biotin carboxylate carrier protein (BCCP), that form N-1,2 carboxybiotin biotin carboxylate carrier protein-BCCP. The reaction is catalyzed by biotin carboxylase (BC). The CO_2 is further transferred to acetyl-CoA by carboxyltransferases (CT). Acetyl-CoA carboxylase (ACCase), a multifunctional enzyme system then catalyzes the formation of malonyl-CoA, from acetyl-CoA [32], which will be subsequently used for *de novo* fatty acid biosynthesis.

4.2 De novo fatty acid biosynthesis

De novo synthesis of acyl chain in the stroma of plastids is catalyzed by a series of enzymatic steps, which collectively forms fatty acid synthase complex (FAS). The series of reactions with the catalyzing enzymes are:

a. Condensation of malonyl-acyl carrier protein (manolyl-ACP) with acetyl-CoA to form 3-ketoacyl-ACP catalyzed by β-ketoacyl-ACP synthase (KAS III).

b. Reduction of 3-β-ketoacyl-ACP to 3-hydroxyacyl-ACP, catalyzed by 3-βketoacyl-ACP reductase.

c. Dehydration of 3-hydroxyacyl-ACP to *trans*-Δ^2-enoyl-ACP, catalyzed by β-hydroxy acyl ACP dehydratase.

d. Reduction of *trans*-Δ^2-enoyl ACP to Acyl-ACP by Enoyl ACP reductase.

This complex also includes an acyl carrier protein (ACP), a cofactor component of FAS to which the growing acyl chain remains esterified. These sequential reactions result in a fully reduced acyl chain, extended by two carbons in each cycle [68] through the sequential round of condensation, reduction, dehydration and second-reduction steps [69]. Repetition of the cycle for six times generates palmitoyl-ACP (16:0-ACP), where the condensation reactions are catalyzed by KAS I. One final cycle reaction between palmitoyl-ACP and malonyl-ACP utilizes KAS II to generate stearoyl-ACP (18:0-ACP). These products are further processed by stearoyl-ACP desaturase (introduce double bonds), plastidial acyltransferases, and acyl-ACP thioesterases (hydrolases). The fatty acyl-ACP thioesterases (FATA and FATB) hydrolyzes the C16-C18 acyl-acyl carrier proteins to generate fatty acids, which are then exported out of the plastids to undergo modifications in the ER [69].

4.3 Elongation of fatty acids

The C16 and C18 compounds, hydrolyzed by acyl-ACP thiosterases are activated into C16- and C18-CoA by long chain acyl-CoA synthetases (LACSs) and exported to the ER. The C16 and C18 acyl-CoA then act as a substrate for fatty acid elongase (FAE) complex, localized on the ER, which adds two carbons successively to form VLCFAs with C26-C34 chains. FAE complex are heterotetramers of independently transcribed, monofunctional proteins. They operate a reiterative cycle of four reactions catalyzed by

i. β-Ketoacyl-CoA synthase (KCS) that catalyze the two carbon condensation to acyl-CoA.

ii. β-Ketoacyl-CoA reductase (KCR) that catalyze the reduction of β-ketoacyl-CoA.

iii. β-Hydroxyacyl-CoA dehydratase (HCD) that catalyze the dehydration of β-hydroxyacyl-CoA.

iv. Enoyl-CoA reductase (ECR) that reduces the enoyl-CoA ultimately leading to VLCFAs [69–71].

4.4 Wax biosynthetic pathways

The elongated products are further modified to produce wax components i.e., to primary alcohols, alkyl esters, aldehydes, alkanes, secondary alcohols, ketones and free fatty acids, via two pathways (i) acyl reduction pathway (generates primary alcohols and wax esters) and (ii) decarbonylation pathway (generates alkanes, aldehydes, secondary alcohols, and ketones).

i. Acyl-reduction pathway: fatty acyl-CoAs are converted into primary alcohols catalyzed by fatty acyl-CoA reductase (FAR) through an intermediate aldehyde [71]. A bi-functional wax synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) enzyme, WSD1 condenses the generated fatty alcohols and C16:0 acyl-CoA into wax esters [26].

ii. Decarbonylation pathway: acyl-CoAs are reduced to aldehyde intermediate by FAR, which are subsequently decarbonylated into alkanes, catalyzed by aldehyde decarbonylase. Stereospecific hydroxylation of alkanes catalyzed by midchain alkane hydroxylase 1 (MDH1) give rise to secondary alcohols, and oxidation of these alcohols form corresponding ketone [32]. Additional hydroxylation and oxidation reactions lead to the esterification of secondary alcohols with fatty acids and formation of diols, hydroxyl ketones and diketones [32].

4.5 Transport and deposition of cuticular waxes

The wax components generated are then transferred from the ER to the plasma membrane (PM) through Golgi and trans-golgi network mediated vesicle trafficking or non-vesicular trafficking [72]. Further, adenosine triphosphate binding cassette (ABC) transporters in the plasma membrane (homodimers and heterodimers) export the wax components to the epidermal surface [73]. Lipid transfer proteins (LTPs) like glycosylphosphatidylinositol (GPI)-anchored LTPs (LTPGs), attached to the outer surface of the plasma membrane are also

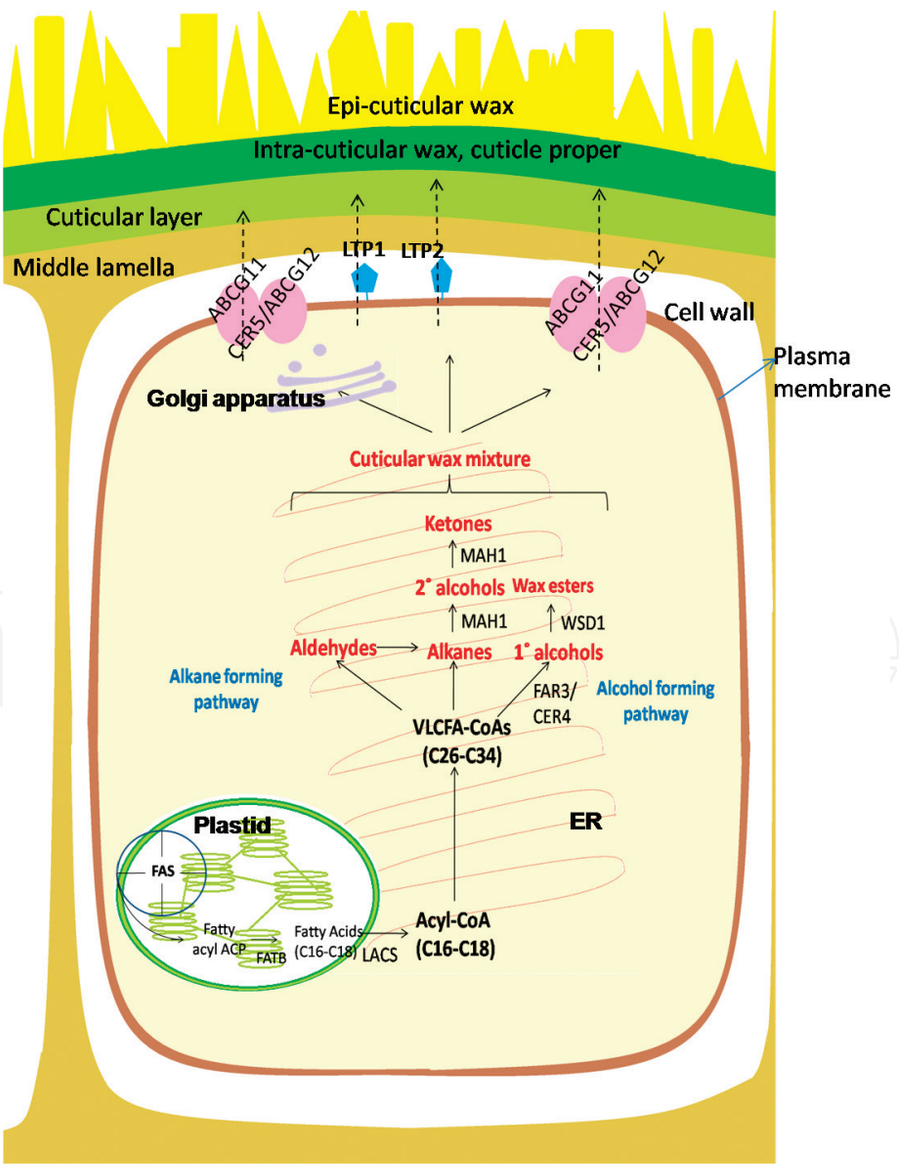

Figure 1.
Schematic representation of wax biosynthesis, transport and deposition in plants.

Gene	Protein type	Role	Reference
Cuticular wax biosynthesis			
ACC1	Acetyl CoA carboxylase	Synthesis of malonyl CoA substrates	[79]
FATB	Acyl acyl carrier protein thioesterase	Supply of saturated fatty acids for wax biosynthesis	[80]
CUT1 /CER6/KCS6	VLCFA condensing enzyme (β-ketoacyl-CoA synthase)	Regulation of VLCFA biosynthesis/elongation of 24C fatty acids	[81]
CER1/CER22	Aldehyde decarbonylase	VLC alkane biosynthesis	[82]
KCS1	β-ketoacyl-CoA synthase	Elongation of 24C fatty acids	[83]
KCS20; KCS2/DAISY	3-ketoacyl-coenzyme A synthase	Required for VLCFA elongation to C22	[84]
LACS1/CER8; LCAS2	Long chain acyl CoA synthetase	Synthetase activity for VLCFAs C20-C30	[85]
KCS9	3-ketoacyl-coenzyme A synthase	Elongation of C22-C24 fatty acids	[86]
WAX2/YRE/FLP1/ CER3	Aldehyde-generating acyl-CoA enzyme	Required for synthesis of aldehydes, alkanes, secondary alcohols, and ketones; biosynthesis of cuticular membrane	[76, 87]
CER10	Enoyl-CoA reductase	Biosynthesis of VLCFA	[88]
CER4/FAR3	Alcohol forming fatty acyl CoA reductase	Formation of C24:0 and C26:0 primary alcohols	[89]
CYP96A15 (cytochrome P450 enzyme)	Midchain alkane hydrolase	Formation of secondary alcohols and ketones (stem cuticular wax)	[78]
WSD1	Wax ester synthase/diacylglycerol acyltransferase	Wax ester biosynthesis	[90]
PASTICCINO2 (PAS2)	3-hydroxy-acyl-CoA dehydratase	VLCFA synthesis in association with CER10, an enoyl-CoA reductase	[91]
KCR1	β-Ketoacyl-CoA reductase	Required for VLCFA elongation	[70]
CER2	BAHD acyltransferase	Fatty acid elongation beyond C28	[92]
CER17 (ECERIFERUM1)	Acyl-CoA desaturase like 4	n-6 desaturation of very long chain acyl-CoAs	[93]
Transport and deposition			
AtWBC12/CER5	ATP binding cassette (ABC) transporter	Transport of cuticular waxes	[94]
LTPG1	Lipid transport protein	Cuticular wax export or accumulation	[74]
ABCG11/WBC11/ DESPERADO	ATP binding cassette (ABC) transporter	Secretion of surface waxes in interaction with CER5	[73, 95]
LTPG2	Lipid transport protein	Cuticular wax export or accumulation	[96]
GLN1, ECH		Vesicle trafficking	[72]

Table 1.
Key genes involved in wax biosynthesis, transport and deposition identified from the model system Arabidopsis.

directly or indirectly involved in wax export [74]. A brief representation of wax biosynthesis, transport and deposition with key genes, is presented in **Figure 1** (adapted from [19, 26, 27, 32, 69, 71]).

Early studies in barley mutants with little or no wax on aerial plant parts, called glossy or glaucous were termed as eceriferum (cer), where cera means wax and ferre means to bear [75]. Subsequently, the wax defective mutants in *Arabidopsis* with bright, shiny, or glossy stems or leaves were also termed as *eceriferum* (*cer*) [76]. The wax locus from maize and *Brassica napus* is termed as *glossy* [68]. With the help of forward genetic screens using wax defective mutants and reverse genetic approaches [77, 78], considerable progress has been achieved in understanding wax biosynthesis, transport and deposition. **Table 1** gives an overlook of the key genes involved in wax biosynthesis, transport and deposition identified from the model system *Arabidopsis.*

5. Regulation of cuticular wax biosynthesis

While the complex wax biosynthesis and transport pathways are well determined, the information on underlying regulatory mechanisms is still fragmentary. There is limited information that these processes and their candidate pathway genes are influenced by developmental factors. The cuticle development is an intrinsic part of cell developmental processes like organ development, cell partitioning, etc. [11]. PAS2, acy-CoA dehydratase, regulating the synthesis of VLCFA during wax biosynthesis in the epidermis is essential for proper cell proliferation during development [97]. Wax deposition is also known to occur in an organ-specific manner during its development and is influenced by diverse environmental conditions as well [17]. The available information on the exact developmental regulation of wax biosynthesis is however, limited. As per evidences from leek (*Allium porrum* L.), wax accumulation and elongation activities are highly induced within a defined and an identifiable region of leaf [98]. The expression of plastidial fatty acid synthase (FAS), FAEs that regulate elongation of long-chain fatty acids in the microsomal membranes and acyl ACP-thioesterases are probable targets of developmental regulation, depending upon the need to produce fatty acid precursor pools [98]. Some of the key genes involved in wax biosynthesis are also affected by defects in the organization of organelles, especially the ER. A mutation of PEX10 (peroxisome biogenesis factor 10) in *Arabidopsis*, which disrupted the ER network, in turn lead to mislocalization of CER4, CER1, SHN1 and WAX2, affecting cuticular wax biosynthesis [99].

There is increasing evidence to show that wax biosynthesis and its pathway genes are regulated at transcriptional, post-transcriptional and translational levels [26, 100]. A wide range of abiotic factors like light, water, temperature, salinity etc., influence wax biosynthesis and deposition. An increase in cuticular wax content is observed in bean, barley and cucumber on exposure to UV-B light [101]. In cotton, enhanced UV-B radiation specifically increased the epicuticular wax load on the adaxial surface of leaves [102]. There is an also an up-regulation of wax biosynthetic genes in salt tolerant rice genotypes under stress [103]. Although the underlying mechanisms have not been well explored in the above conditions, there is sufficient information on the influence of drought or moisture stress on wax biosynthesis in plants. A significant increase in wax load in *Arabidopsis* plants subjected to water stress is indicative of its regulation under drought [17]. In crops like rice, wheat, tobacco, alfalfa, peanut and cotton, etc., an increase in cuticular wax accumulation was observed under moisture stress condition [104]. Drought induced accumulation of wax biosynthesis is positively correlated with drought tolerance in crops like oat, rice, wheat and forage crops, etc. [104–107].

The transcript levels of several genes involved in wax biosynthetic pathways are regulated in response to abiotic stresses. FAR5, a fatty acyl CoA reductase, in wheat responsible for accumulation of long chain primary alcohols of C26:0, C28:0 and C30:0 are regulated by drought, ABA and cold [108]. The transcripts of KCS2/DAISY, a 3-ketoacyl-coenzyme A synthase required for the elongation of VLCFA are up regulated under water deficit conditions [84]. Osmotic stress induces the expression of CER1, that regulates alkane biosynthesis; while the over expression of CER1 increased susceptibility to bacterial and fungal pathogens [109]. Hypoxia is also known to affect total wax loads on *Arabidopsis*. The expression of KCS, KCR1, ECR/CER10 and PAS2, components of fatty acid elongase complex in *Arabidopsis* stem and leaves is affected which in turn affects the production of VLCFA precursors of wax biosynthesis. The wax synthesis genes like MAH1, CER3, CER4, WSD1, etc., and several genes associated with wax and lipid transport are also affected by hypoxia [110]. There is also indication on the regulation of wax biosynthesis in response to cold. Acteyl-CoA carboxylase plays the essential role for cold acclimation in *Arabidopsis*. In sensitive to freezing3 (*sfr3*) mutants, with a missense mutation in ACC1, the long chain components of leaf cuticular wax were reduced and there was inhibition on the wax deposition on inflorescence stem, which rendered the plants sensitive to cold stress [111]. Wax biosynthesis is also reported to be regulated in response to carbon dioxide (CO_2) concentration. This is mediated by HIC (High Carbon Dioxide), a gene encoding a 3-keto acyl coenzyme A synthase (KCS)-an enzyme involved in the synthesis of very-long-chain fatty acids that influences stomatal development in *Arabidopsis* [112].

With the identification of several transcription factors (TFs), transcriptional regulatory mechanisms are considered to be a major contributor for the wax biosynthesis [113]. WIN1/SHN1 (WAX INDUCER 1/SHINE1) is a TF from AP2/EREBP family initially reported to regulate cuticular wax and then cutin biosynthesis by regulating the expression of *CER1*, *KCS1*, *CER2*, *LACS2*, *GPAT4*, *CYP86A4*, *CYP86A7* and *HTH*-like genes [114]. SHN1 overexpression increased drought tolerance in *Arabidopsis* [115]. Wax synthesis regulatory gene 1 (WR1) from rice [116] and SHN1 from wheat [117], both homologs of WIN1/SHN1 from *Arabidopsis* also reduced water loss and improved drought tolerance. Transcriptional repression by diurnally controlled DEWAX2 is another important for regulator of wax biosynthesis in *Arabidopsis*. Compared to wild type, the total wax loads in *dewax2*, were increased by 12 and 16% respectively in rosette and cauline leaves [118, 119]. Another candidate from AP2/ERF TF family, WRINKLED4 (WRI4) positively regulates wax biosynthesis in stems. *wri4* mutants expressed 28% reduction of total wax loads in stems, although siliques and leaves were unaffected. Hence WRI4 act as a transcriptional activator to regulate the expression of LACS1, KCR1, PAS2, ECR and WSD1, to maintain the levels of 29C long alkanes, ketones and secondary alcohols in stems [113]. MYB94, regulate the expression of wax biosynthetic genes like WSD1, KCS2/DAISY, CER2, FAR3 and ECR to activate cuticular wax biosynthesis and is up regulated by drought and ABA. This also conferred tolerance to drought stress in *Arabidopsis* and *Camelina* [120]. MYB96, an ABA responsive TF also regulates wax biosynthesis under drought [121]. In *Camelina*, MYB96 activated the expression of wax biosynthetic genes KCS2, KCS6, KCR1-1, KCR1-2, ECR, and MAH1 which resulted in high levels of alkanes and primary alcohols and improved drought tolerance [120]. MYB96 acts as a component of plant disease resistance, through salicylic acid mediated signaling [122]. Both MYB94 and MYB96 share a common region containing MYB consensus motifs in the promoter of their target wax biosynthetic genes [123]. Hence MYB94 and MYB96 have an additive role on plant cuticular wax biosynthesis and under drought and ABA conditions.

In addition, to transcriptional regulation, wax biosynthesis is regulated by other events. Expression of CER3/WAX2/YRE, an aldehyde-generating acyl-CoA enzyme

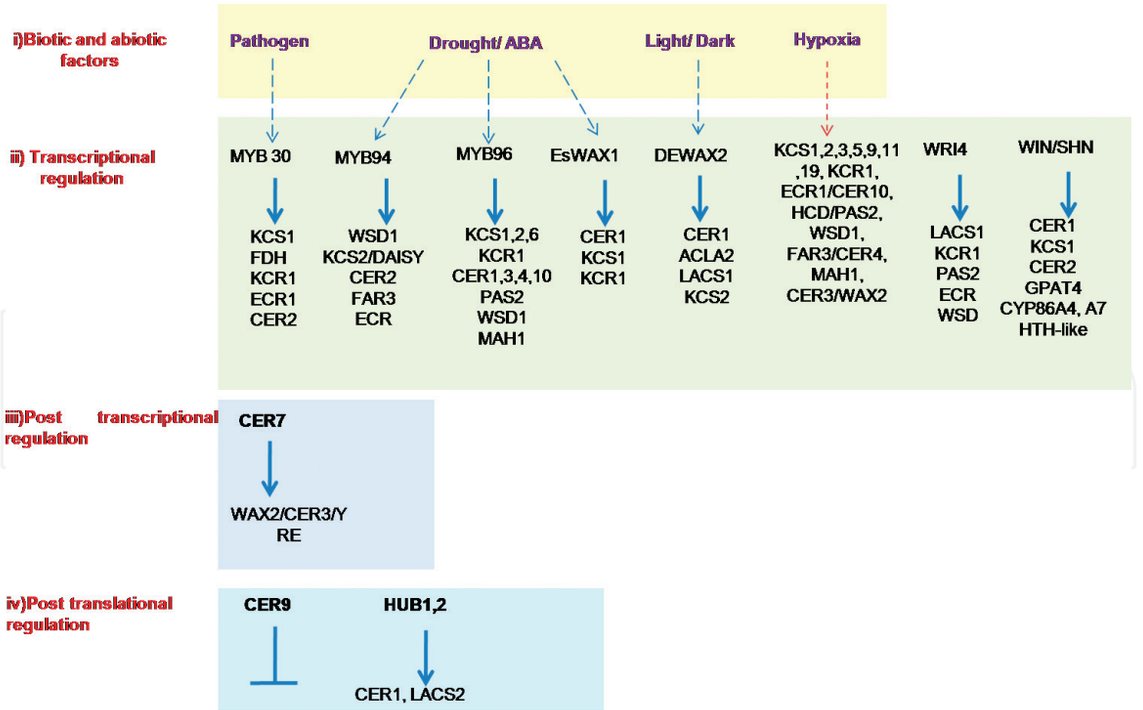

Figure 2.
Brief representation of the key regulatory events in wax biosynthesis and their targets.

in the wax biosynthetic pathway is regulated by CER7, a core RNA processing and degrading exosomal subunit. CER7 regulates WAX2 transcript levels by degrading a specific mRNA species encoding its negative regulator [124]. Many of such regulators have been identified from model systems as well as crop species and a brief overview of the key regulatory events and their targets has been presented in **Figure 2**.

6. Cuticular wax trait in imparting stress resistance

Under field conditions, crops encounter multiple biotic and/or abiotic stresses simultaneously at different stages of developments. Cuticular waxes have a direct role in multiple stress tolerance in crops [109]. In cucumber, wax biosynthesis has been shown to have key roles in influencing the plant responses to biotic as well as abiotic stresses [125]. In sorghum, genes regulating leaf waxes have critical role in regulating tolerance to drought and heat stress [45]. Considering the relevance of cuticular waxes under diverse biotic as well as abiotic stressful conditions, as discussed above and under combined stress conditions, it can be an ideal trait to tackle multiple stresses in crop plants.

6.1 Biotic stresses

6.1.1 Pathogens

Being the outermost layer of plant cuticle, the epi-cuticular wax can serve as a first line of physical defense against pathogens and herbivores. However, increasing thickness and hydrophobicity of the cuticle through over-deposition of the wax may not necessarily increase the resistance of the plant against biotic stresses. The composition and structure of wax in the cuticle can constitute the source of signals for the foreign invaders and for the plants themselves. Thus, the roles of cuticular wax could be multifunctional and can vary not only for various plant species but

also for different kinds of pathogens. Functional study of the *DEWAX* gene, a negative regulator of wax biosynthesis in *Arabidopsis*, is a good example of this complexity. The *dewax* mutant line in *Arabidopsis*, with increased epicuticular wax and decreased cuticular permeability, showed susceptibility to the fungal pathogen *Botrytis cinerea*, but resistance to the bacterial pathogen *Pseudomonas syringae* [126]. Moreover, *DEWAX* overexpressing lines in *Arabidopsis* and *Camelina* showed inverse defense modulations to *B. cinerea* and *P. syringae* as compared to *dewax* mutant in *Arabidopsis* [126].

Wax and cutin components in the plant cuticle could function in pattern- and effector-triggered immunity (PTI and ETI) and could serve to generate local and systemic acquired resistance against numerous pathogens [127]. During plant-pathogen interaction, the plant cuticle can be affected by enzymes synthesized and secreted by the pathogens. Many fungal pathogens synthesize and secrete hydrolytic enzymes (for example, cutinases, esterases and lipases) at the early stage of infection that directly target the cuticle [128–131]. *Fusarium oxysporum* secretes cutinases that degrade cutin layers in the cuticle and generates cutin monomers that support fungal adherence to the host plant and facilitate the initiation of infection [128]. Hexadecanediol, a cutin component in rice can facilitate spore germination and differentiation for pathogenic fungi *Magnaporthe grisea* and *B. cinerea* [55]. Presence of a very-long-chain C_{26} aldehyde (a wax component) was important for the barley powdery mildew fungus (*Blumeria graminis*) to initiate infection in host plant species. Germination and appressorial differentiation of *B. graminis* were strongly prohibited in aldehyde free *glossy11* mutant in corn. Spraying of *n*-hexacosanal (C_{26}-aldehyde) or wax preparation from wild-type corn can restore the conidial formation and differentiation [59].

Plant can also recognize the attachment of pathogens and activate defense responses against them, in which pathogen-infection generated plant products, such as cutin monomers or cell wall oligosaccharides, can act as signaling molecules [132]. Defense responses in plants are often manifested as alternations of the cuticle. *Colletotrichum acutatum* infection in citrus resulted in increased lipid synthesis in the epidermal cell and increased deposition of those lipids in cuticle, the process eventually changes the structure of the cuticle [133]. Cuticular biosynthesis was also found to be up-regulated in tomato fruit following infection by fungal pathogen *C. gloeosporioides* [134].

Cuticular permeability plays a vital role in almost all plant-pathogen interactions. A more permeable cuticle can lead to either resistance or susceptibility to pathogens. Elevated deposition of cuticular wax as well as the presence of hydrophobic wax components (e.g., very-long-chain alkanes or ketones) can make a cuticle less permeable. Mutation or overexpression of genes that diminish biosynthesis of various wax components can generate the opposite effect. There are number of wax-deficient mutant and transgenic lines in *Arabidopsis* and other plant species with diminished cuticular permeability showed resistance to the fungal pathogen *B. cinerea* [34, 127]. However, the phenomenon is not true for all wax deficient plant lines. Wax and cutin deficient *acp4* and *gl1* mutants in *Arabidopsis* displayed increased sensitivity to *B. cinerea* [135, 136]. Mutations in SHINE transcription factors in other studies also showed alteration in cuticular wax accumulation, and susceptibility to *B. cinerea* infection [137, 138].

6.1.2 Insects and herbivores

Epicuticular wax also plays important roles in plant interaction with insects and herbivores. Flowering plants have evolved with cuticular wax of various forms, sizes and structures that are either enabling the attachment and movement of

pollinating insects, or reducing the attachment of herbivorous insects and pests on the plant surfaces. Reducing the attachments of herbivores on plant surfaces is a part of a plant defense strategy against herbivores.

Most plant body surfaces are covered with a two-dimensional (2D) epicuticular wax film of various thicknesses. In many species, wax film is protrudes with three-dimensional (3D) wax crystals. Wax crystals can generate various shapes as revealed by electron microscopic analysis, such as rodlets, threads, platelets and tubules [61]. The complexity of these various shapes originates from the molecular self-assembly of various wax components, in which morphology of those crystals is also correlated with the presence of specific chemical components in the wax [139, 140]. Many experimental studies and reports from various plants species (for example, from genera *Eucalyptus*, *Pisum*, *Brassica*) have shown that 3D wax crystals have protective functions against insects, in general, including the herbivorous insects [141]. Studies with *Eucalyptus* species in canopy found that glaucous juvenile leaves containing high quantities of wax crystals were less prone to herbivorous infestation as compared to the glossy adult leaves [142]. Feeding rates of flea beetles, *Phyllotreta cruciferae*, on low-wax glossy (*eceriferum, cer*) *Brassica napus* mutant lines were much higher as compared to the wild-type *B. napus* [143]. Cuticular surfaces with wax crystals also interferes with the attachment, locomotion and foraging behavior of predatory insects and parasitoids [65, 144]. *Pisum sativum* lines with higher prevalence of crystalline epicuticular wax (CEW) were found more favorable for four predatory coccinellid species to attach, move and consume more aphids as compared to the *P. sativum* mutant line with reduced CEW [145]. Flowering stems with high CEW of numerous other plant species (for example, species under the genera *Salix*, *Hypenia*, *Eriope*) often generate slippery surfaces that prevent the movement of nectar robbers, ants and other plant pests [141, 146].

Several hypotheses have been proposed and tested on the mechanisms of wax crystal inhibition of insect attachment inhibition: (i) roughness hypothesis; (ii) contamination hypothesis; (iii) fluid absorption hypothesis [141]. Wax crystals, in general, generate a micro-rough surface on the cuticle that may prevent adhesive pads of the insects to stick, preventing them to successfully attach to the plant surface [144, 147, 148]. Contamination hypothesis proposed that detached wax crystals of the cuticular surface of some plants can adhere to the insect attachment organs (e.g., adhesive pads), contaminate those, and as such subsequent insect attachment becomes challenging and unsuccessful [147–149]. Adhesive pads of many insects secret fluids, which can also enhance wax crystal contamination to attachment organs. Fluid secretion from the adhesive pads are supposed to help insects to pursue successful attachment to the plants. However, there is evidence certain plant species have crystalline wax coverage that can absorb the fluids secreted by the adhesive pads and prevent the insects to successfully attach to the cuticle [150, 151].

The study of cuticular wax involvement in biotic stress resistance is complex with a multitude of organisms spanning insects to disease. The story is still not clear and field situations in which interactions between organisms and abiotic stresses and the role of cuticular wax needs to be evaluated. Nevertheless, certain consistencies are evident in that permeability of the cuticular layer appears to be important in pathogen invasion and wax crystals play an important role in insect intervention by the cuticular layer. These areas of research merit further investigation.

6.2 Abiotic stresses

As mentioned above, abiotic stresses such as drought, extremes of temperatures, salinity, etc., cause significant losses in crop productivity. Since most of the

stresses occur simultaneously, crop breeders are looking for traits contributing for multiple stress resistance. From this context, cuticular wax can serve as ideal trait. Drought stress, a major abiotic stresses in tropical regions, influences the biosynthesis and composition of cuticular wax in crops [27]. The importance of cuticular wax in desiccation tolerance is evident that, compared to gymnosperms and angiosperms, many early extant plants such as ferns, and horsetails are more sensitive to dehydration [152]. In crops like pea, cuticular wax load increases when subjected to drought stress [46]. In rice, *gl1*-1/wsl2 and *gl1-2* loss-of-function mutants with reduced wax load exhibited sensitivity to drought compared to the wild type plants [104, 153]. Drought stress is known to increase the wax content and alter composition of cuticular wax in many plants such as pea [46], *Arabidopsis* [17, 115], tobacco [154], alfalfa [155]. Significant correlations between the wax content and yield, drought tolerance and water-use efficiency have been reported in different crops such as sorghum [38], barley [156], rice [41], and wheat [157, 158]. These reports demonstrate that less wax or non-waxy crops/genotypes are sensitive to desiccation with poor drought-tolerance compared to the crops having more cuticular wax [105]. The existing evidences suggests cuticular wax is responsible for reducing non-stomatal transpiration by increasing cuticular resistance [43]. The cuticular waxes also have roles in imparting resistance to salinity stress, mainly by regulating residual transpiration. A significant negative correlation observed between residual transpiration and total wax content, reports residual transpiration could be a fundamental mechanism by which plants optimize water-use efficiency under salinity stress [43]. As discussed above, wax accumulation also correlated with high temperature resistance in plants [44]. Leaf surface waxes help to maintain cooler canopy in sorghum under heat stress [45]. The cuticular waxes can further help in protecting plants from high light stress [101]. The cuticular wax has a role in protecting plants from excessive ultraviolet (UV) light and there are reports indicating that elevated UV-B radiation can affect plant cuticular wax formation [101, 159, 160]. Based on the existing information, as mentioned above, cuticular wax, can be treated as the first protective layer and an important trait contributing for both biotic and abiotic stresses.

7. Attempts by crop biologists to manipulate cuticle traits

7.1 Breeding

Identification of genomic regions contributing wax traits is crucial in manipulating wax characteristics using breeding approaches. In rice, quantitative trait loci (QTL) linked to the leaf epi-cuticular layer was identified corresponding to EM15_10-ME8_4-R1394A-G2132 region on chromosome 8 [161]. In sorghum, a crop with the ability to produce profuse amounts of EW, BLOOM-CUTICLE (BLMC) locus from chromosome 10, was identified to account for profuse wax production. BLMC region corresponds to approximately 153,000 bp with three co-segregating markers and an acyl CoA oxidase with seven other putative candidates. BLMC mutation affected C28-C30 free fatty acid fractions and hence cuticle properties in culm and leaves, disrupted EW production and increased plant death rating in field at anthesis [162]. With the genetic analysis of F2 population from HUAYOU2 (P1 X M36), BoWax1 locus (*Brassica oleraceae* Wax 1) is identified to be controlling glossy green trait in cabbage, due to a deletion mutation of two nucleotides in the cDNA of Bol013612 of HUAYOU2. BoWax1 locus maps to chromosome CO1 [163]. The wax biosynthetic pathway genes identified in pearl millet were co-located to the QTL

controlling biomass production under early drought stress and stay green traits [164]. Targeted breeding using the modern molecular breeding for this trait would be useful.

7.2 Transgenic

With the elucidation of wax biosynthetic pathways and identification of key regulators, attempts were made in crop plants to engineer cuticle properties and to enhance stress tolerance traits. One of the early reports in engineering wax traits and thereby improved stress tolerance was from *Medicago sativa* (alfalfa), a forage legume. WXP1, a transcriptional regulator from *Medicago truncatula*, upregulated by drought, cold and ABA, was over expressed in alfalfa, which significantly increased the leaf cuticular wax load, mainly contributed by the C30 primary alcohol. The transgenic plants exhibited enhanced tolerance to drought and rapid recovery under rehydration [155]. Over expression of SlSHN1, a close homolog of the WIN/SHN gene from *Arabidopsis*, in tomato using constitutive CaMV 35S promoter improved drought tolerance, with higher cuticular wax deposition on leaf epidermal tissue. The transgenic plants displayed delayed wilting, improved water status and reduced water status [165]. MYB96, a transcriptional regulator over-expressed in *Camelina*, an emerging biofuel crop, which generated plants with enhanced drought tolerance. The expression levels of CsKCS2, CsKCS6, CsKCR1-1, CsKCR1-2, CsECR, and CsMAH1 were highly upregulated in the transgenic plants which resulted in a significant increase in the deposition of epicuticular waxes and total wax loads. This gives an option to cultivate the crop on marginal lands to produce renewable biofuels and bioresource [120]. It was further demonstrated that ectopic expression of DEWAX, a negative regulator of cuticular wax biosynthesis increased tolerance to *Botrytis cinerea* in *Camelina* [126]. A study from groundnut by over-expressing the KCS1 gene from a drought tolerant genotype improved cuticular was load and drought tolerance in a susceptible genotype [166]. Likewise, several of such regulators have been identified from model systems as well as crop species and used for engineering crop plants to enhance stress tolerance.

8. Options for manipulation of wax traits for individual and/combined stress tolerance

In crop plants, due to the nature of combined stressors interactions, the stress effect is not always additive [3]. While working with glossy mutants of *Zea mays* (*gl4*), an enhanced colonization of bacteria, was observed leading to more leaf blight pathogen growth compared to the wild type [167]. The thin cuticle provided leaf blight pathogen, an easy access to nutrient and water in *gl4* mutant indicating that cuticular wax thickness is a useful trait to identify plants' resistance to combined stressors. Additionally, wax layer structure and composition are equally important in conferring defense mechanisms. As rightly pointed in Ref. [1], such combined studies allow us to understand the shared and specific effects of biotic and abiotic stressors.

Wild relatives and landraces have long been recognized as a source of genes for breeding major field and horticulture crops. During domestication of wheat, tomato, rice, soybean and corn, yield was the focus trait. This in turn narrowed the genetic diversity for other biotic and abiotic stressors [168]. For example, during domestication of modern wheat, due to a phenotyping bottleneck a largely

overlooked drought trait in wheat breeding program is glaucousness [169]. Such beneficial allelic variants lost in cuticle related traits can be introgressed back by crossing an elite line with its wild relatives. Apart from genetic diversity, a mutation population (EMS or gamma irradiation) provides an alternative avenue to target crop improvement via selection of cuticle-associated trait variations [170]. In fleshy tomatoes, a mutant line underlying for *delayed fruit deterioration* (DFD), is characterized for minimal transpirational water-loss and enhance post-harvest shelf life [171]. A recent alternative for trait manipulation is CRISPR-Cas9 system which is a precise gene-editing technology. This new method accelerates the evaluation of beneficial cuticle-associated alleles in different genetic backgrounds [172]. In similar lines, small RNA based transgenic strategy is also emerging as a molecule of choice to deal with combined biotic and abiotic resistance in crops [173].

9. Conclusion

There is sufficient evidence to argue that cuticle and cuticular waxes are involved in the regulation of multiple biotic and abiotic interactions. The cuticular wax can be treated as an important trait contributing for multiple stress resistance. Concerted efforts have been made to elucidate the synthesis and deposition of cuticular waxes in plants. Further analysis of the key regulatory steps involved in the formation of cuticular waxes, and also the role played by diverse types of wax components and structures in stress response is needed. This information could be incorporated in crop improvement programs (via marker assisted selection for wax genes). Since there are promising options emerging to analyze the cuticular wax trait using modern synchrotron technology [174] as well as now widely recognized techniques to observe ice propagation in real time across the cuticle [175] crop breeders have the potential to improve their efficiency of selection based on these traits. Recent progress in genomics can substantially help major field and horticulture crops to buffer the impacts of climate change. In addition, new genome-editing technologies will provide interesting tools to characterize and engineer waxes in crops. Unraveling key regulators and network partners of surface wax synthesis would aid in targeted manipulation of the trait using modern biotechnological applications. There are options to analyze the cuticular wax trait using modern non-destructive approaches. Crop breeders can use these tools to improve their efficiency of selection for the trait, and effectively pyramid the trait in elite genotypes to combat combined stresses.

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