CHAPTER 5

Biology and Biochemistry of Ethylene

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5.1 INTRODUCTION

Among the plant hormones, ethylene is unique for several reasons, mainly linked to its simple two-carbon gas chemical nature: C_2H_4. Both its structure and chemical state imply that if on one hand ethylene is normally synthesized in its site of action, on the other it can also diffuse and accumulate in localized areas, acting as a long-distance signaling molecule.

The identification of ethylene as a plant hormone is preceded by a long and surprising story of agricultural and cultural practices involving the unaware utilization of this hormone already more than 2,000 years ago, when in ancient China incense smoke was used to accelerate ripening in pears. We now know that the combustion of incense, which is derived from plants, generates the small hydrocarbon ethylene and that plants produce this hormone to promote fruit ripening. Ancient Egyptians used to gash figs, as pointed out by some archeological finds, to induce ethylene production and trigger ripening, even in the absence of the insect pollinator.

The history of ethylene research starts in 1896, when, at the Botanical Institute of St. Petersburg, Dimitry Neljubow observed that the growth of his pea seedlings was affected by the gas lamps that illuminated the laboratory. Dark-grown seedlings displayed a triple response of reduced elongation and radial swelling of the hypocotyl, short roots, and enhanced apical hook formation,

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allowing Neljubow to identify ethylene as the causal agent, and to establish that plants are able to sense this compound and respond with a well-defined biological effect. A few years later, in 1934, Richard Gane added another milestone to the history of this fascinating gas. He quantified the ethylene produced in one month by 30 kg of ripening apples and demonstrated definitely that plants are able to synthetize ethylene. Although the development of new methods of detection in the 1950s allowed to improve the knowledge of ethylene physiology, the biosynthetic pathway of this hormone was revealed only two decades later, by Shan Fa Yang (see Section 5.2.1). With the advent of molecular biology techniques and the adoption of *Arabidopsis thaliana* as a model plant, the whole of the perception and signaling mechanisms were definitely disclosed.

Several studies carried out in the following years, not only in model species but also in crops, greatly improved the understanding of the biological effects of this hormone. Ethylene plays either pivotal or accessory roles in many diverse processes, ranging from seed germination, adventitious root formation, interactions with microbes, and pathogen-mediated cell death, to leaf and flower senescence and abscission, responses to stress, up to fruit ripening and senescence. One of the most difficult aspects of ethylene research deals with the high specificity of its action in many of its roles. For example, ethylene inhibits cell elongation in the dark, whereas it can promote it in light or it stimulates elongation in plants grown in low-phosphorous conditions, but has an inhibitory role in high phosphorous. Moreover, to further complicate its study, ethylene has extensive synergistic, additive, and/or antagonistic interactions with the other phytohormones.

Despite the wide involvement of this gaseous hormone in several processes of a plant's life, the most important commercial potential of ethylene research deals with fruit ripening and the postharvest physiology, especially for the so-called climacteric fruits. This type of fruits, including, for example, tomato, apple, pear, peach, and banana, displays a ripening-related burst of both respiration (oxygen consumption and carbon dioxide production) and ethylene production, termed the "ethylene/respiratory climacteric." Conversely, nonclimacteric fruits (such as, e.g., citrus, grape, and strawberry) do not show such a climacteric rise, also keeping carbon dioxide and ethylene production at basal levels during ripening. When mature climacteric fruit are treated with exogenous ethylene, an acceleration of ripening occurs, with an anticipated respiratory climacteric rise and the upregulation of ethylene-dependent genes, including those responsible for the biosynthesis of the hormone resulting in the autocatalytic production (see Section 5.2). This physiological feature leads to an irreversible activation and promotion of ripening. Nonclimacteric fruits do not require ethylene action for normal ripening to take place, however they still maintain the ability to respond to exogenous ethylene through the upregulation of a subset of ripening

processes. In fact, when treated with exogenous ethylene they display some physiological responses (such as a temporary increase in respiration, degradation of chlorophyll) but they do not produce autocatalytic ethylene, thus the physiological effects are limited and dependent on the presence of the gas in the environment.

During ripening, ethylene stimulates its own biosynthesis and induces the expression of many genes associated with the processes characterizing the ripening syndrome. Some of these genes are involved in the synthesis of pigments, aromas, and flavors, sugar production from starch, and cell wall changes associated with fruit softening. Consequently, optimal conditions in the preharvest phase and remarkably during the postharvest (transport and storage) have significant implications for human health and wellbeing, as they may strongly affect the final quality of the fresh fruits.

A considerable effort has been dedicated to studying the role of ethylene in fruit development and ripening, and a huge amount of research is still ongoing on this topic, with significant impacts on the global agricultural marketplace. Researchers have tried to modulate ethylene biosynthesis and perception/response either to delay or block ripening, thus improving fruits' tolerance to storage and long-distance transport, or to accelerate ripening to anticipate harvesting. Different approaches have been adopted, either through chemical tools or genetic engineering, but all of them depend on a successful research strategy and the achievement of new knowledge about the regulation of ethylene biosynthesis, perception, and signal transduction. A detailed understanding of these aspects may also allow the exploration of the natural variability to identify interesting genotypes to be used to constitute new superior varieties.

5.2 ETHYLENE BIOSYNTHESIS, PERCEPTION, AND SIGNAL TRANSDUCTION

5.2.1 The Ethylene Biosynthetic Pathway: Yang's Cycle

The biosynthesis of ethylene began to be elucidated relatively late, not only because of the lack of technical tools able to accurately quantify ethylene, but also due to both the instability and the low active levels of the key enzymes involved in the last steps of the pathway. The first "eureka moment" (late 1950s) was the discovery that ethylene synthesis requires oxygen, implying that at least one enzyme with oxidase activity was needed. Later, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was identified, opening the way to the following studies. ACC is synthesized by the enzyme ACS (ACC synthase) from the amino acid methionine, which is first converted to S-adenosyl-methionine. ACS, therefore, catalyzes the conversion of S-adenosyl-methionine to ACC and methylthioadenosine. In the presence of oxygen, ACC

is converted to ethylene, CO_2, and HCN, thanks to the action of the enzyme ACC oxidase (ACO). Methylthioadenosine resulting from ACC synthesis is used to regenerate methionine following the steps described in the so-called Yang's cycle (Fig. 5.1).

The accumulation of ACS represents the rate-limiting step of ethylene biosynthesis and this feature is used by plants to finely tune the levels of the hormone. This enzyme acts as a dimer and is encoded by a multigene family with the different members differentially expressed in diverse cell types, tissues, and

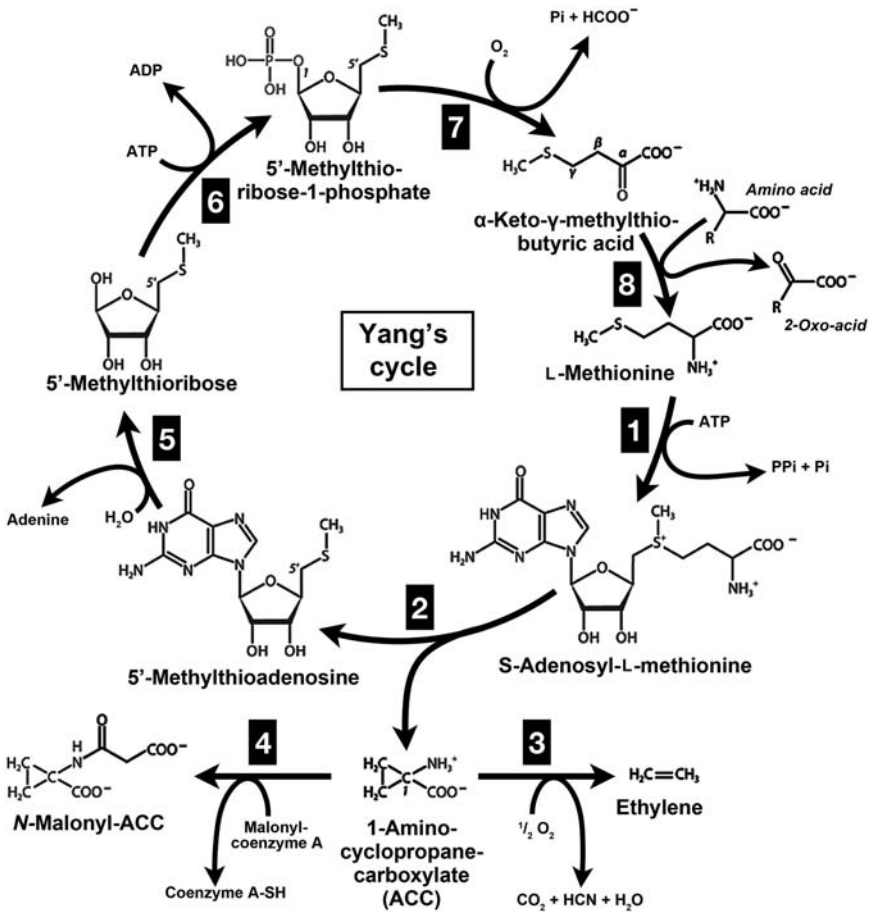

FIGURE 5.1

Yang's cycle of ethylene biosynthesis and regeneration of its precursors. The formation of S-adenosyl-methionine is catalyzed by S-adenosyl-methionine synthase (1) from methionine and ATP. Aminocyclopropane-carboxylate (ACC), the precursor of ethylene, is then produced by ACC synthase (ACS; 2) along with methylthioadenosine (MTA). ACC oxidase (ACO; 3) catalyzes the final step of ethylene biosynthesis, using ACC as a substrate and generating carbon dioxide and cyanuric acid as secondary products. MTA recycling allows a constant level of methionine, even under conditions of high ethylene production (steps 5–8) (5, MTA nucleosidase; 6, MTR kinase; 7, transaminase; 8, spontaneous reaction). ACC malonylation to malonyl-ACC deprives ethylene biosynthesis of ACC (ACC *N*-malonyl transferase; 4).

organs. Therefore, according to the ACS genes expressed and their levels of expression, their combination into a dimer may have different enzymatic activity and stability, thus determining the final levels of ethylene. This regulatory aspect becomes functionally relevant when the mode of ethylene biosynthesis shifts from autoinhibitory (the so-called "system 1" responsible for basal ethylene production before the climacteric burst) to autocatalytic ("system 2" responsible for climacteric ethylene production), thus allowing the climacteric burst. The change from system 1 to system 2 is allowed by the upregulation of specific ACS with a high catalytic activity and stability. Application of propylene (an analogue of ethylene) can trigger an increase in respiration in both climacteric and non-climacteric fruits but a propylene-mediated induction or rise in endogenous ethylene production occurs only in climacteric fruit, mainly due to the upregulation of the same ACS genes naturally induced during the system 1 to system 2 transition.

5.2.2 Ethylene Perception and Signal Transduction

The adoption of *Arabidopsis thaliana* as a model plant gave a significant acceleration to ethylene research, in particular regarding the studies on ethylene perception and signaling. The triple response represented a very rapid and easy way to screen for mutants with an impaired ethylene response, allowing the identification of the genes encoding its receptors several years before any plant genome was sequenced.

Ethylene is perceived by a family of receptors localized at the endoplasmic reticulum, with the ethylene binding site lying within the membrane and binding ethylene with the aid of a copper cofactor. Due to its chemical features, ethylene not only freely crosses cell membranes, but actually solubilizes and freely diffuses within, thus facilitating its binding to receptors. The first ethylene receptor, ETR1 (EThylene Resistant 1), was isolated and identified by Anthony Bleecker's group. The sequence of the corresponding gene is very similar to the two-component family of histidine protein kinase receptors that are highly prevalent in prokaryotes and rare in eukaryotes. ETR1 was the first protein unambiguously identified as a hormone receptor in plants. Its discovery was immediately followed by the identification of another receptor, ETR2, and the remaining three ethylene receptors in *Arabidopsis*.

The most important feature of the ethylene perception mechanism deals with the negative regulation exerted by the receptors, as confirmed by plants that are heterozygous for wild-type and dominant mutant alleles and show an ethylene-insensitive phenotype. In the absence of ethylene, wild-type receptors block the ethylene response and when bound to ethylene this block is released, thus triggering the response to the hormone. On the other hand, the mutated receptors keep the ethylene response blocked, even in the presence of the ligand.

Ethylene receptors can be divided into two families, according to their structural and functional characteristics. In *Arabidopsis*, subfamily I receptors (characterized by three membrane-spanning domains and intact HK domains) play a more prominent role in the ethylene response than those belonging to subfamily II (four membrane-spanning domains and degenerate HK domains), as demonstrated by loss-of-function mutants. Ethylene receptors function as dimers, but may also associate with additional proteins, such as the downstream components Constitutive Triple Response 1 (CTR1) and Ethylene INsensitive 2 (EIN2), or be affected by accessory mediators, such as Reversion To Ethylene sensitivity 1 (RTE1;Green-Ripe,GR, in tomato), the latter exerting a negative regulation on the perception of the hormone (Fig. 5.2).

The signaling pathway of ethylene downstream of the receptors has been elucidated and most of the components identified and assembled in the first draft of the ethylene signal transduction. The first two key elements are CTR1 (the name derives from the constitutive triple response of its mutant) and EIN2. The former is a negative regulator of the ethylene response and is active when bound to the free receptor. Its activity is due to an mitogen-activated protein kinase kinase kinase (MAPKKK) domain that is inactivated when ethylene binds its receptors. The following element, EIN2, is a positive regulator and its function was elucidated quite recently (2012) by three independent groups. EIN2 is normally phosphorylated by CTR1 in the absence of ethylene, to be then degraded through the proteasome. When CTR1 is inactivated by ethylene binding to receptors, EIN2 is stabilized and its C-terminal end cleaved, acting as a mobile signal to the nucleus (Fig. 5.3).

Although a link between the presence of the C-end of EIN2 in the nucleus and the following molecular events is still missing, the sequence of events leading to the final transcriptional response to ethylene is well established. In the presence of the hormone, EIN3 and EIL1 (EIN3-like 1) transcription factors are stable and positively regulate the expression of ethylene-related genes. In the

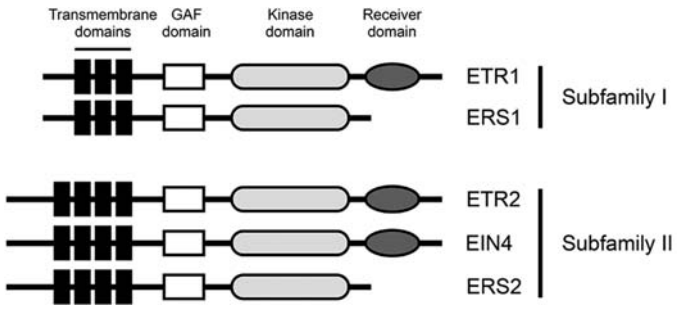

FIGURE 5.2

Ethylene receptors are encoded by a multigene family that can be grouped into two main subfamilies according to the number of hydrophobic domains at the N-terminal. Subfamily I includes EThylene Resistant 1 (ETR1) and Ethylene Response Sensor 1 (ERS1), while subfamily II comprises EThylene Resistant 2 (ETR2), Ethylene INsensitive 4 (EIN4), and Ethylene Response Sensor 2 (ERS2). The ERS receptors differ for the absence of the C-terminal receiver domain.

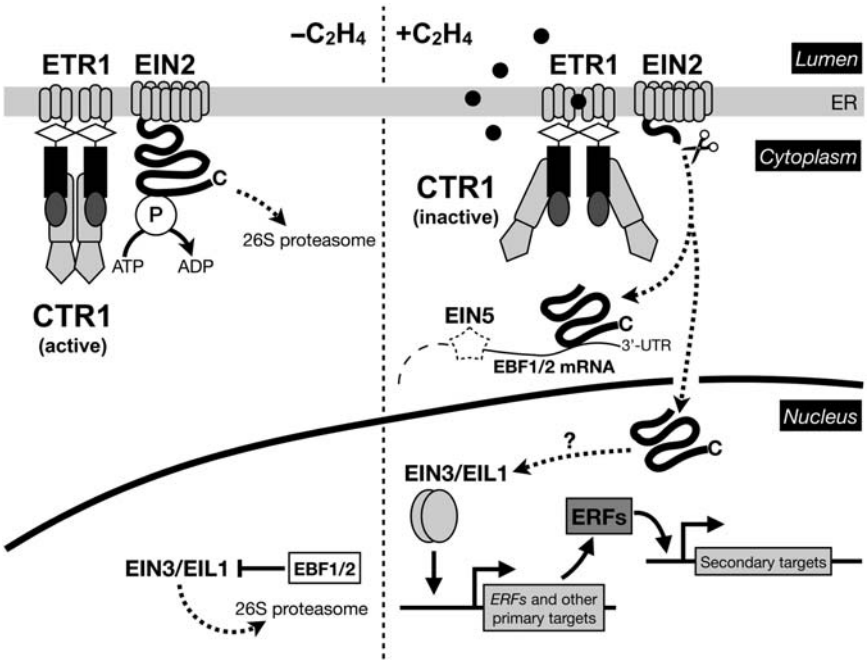

FIGURE 5.3

The ethylene signal transduction requires the involvement of several elements, among which CTR1, EIN2, EIN3, and the EIN-like (EIL) proteins are the most important. Constitutive triple response 1 (CTR1) is the first element downstream from the receptors, working as a negative effector that leads to proteasome-mediated degradation of EIN2. In the presence of ethylene, CTR1 is deactivated and the transduction pathway is unlocked, with the C-terminus of EIN2 that is cleaved, thus entering the nucleus to trigger ethylene response. EIN3 and EIL1 transcription factors are activated and bind the promoters of ethylene-responsive genes as a homodimer, thus promoting their transcription. Among these genes, the *ERFs* encode transcription factors that promote in turn the transcription of secondary target genes (described in Section 5.3), thus coordinating the downstream ethylene response. The levels of EIN3 are also regulated by 26S proteasome-mediated degradation due to targeting by specific F-boxes (EBF1/2) belonging to poly-ubiquitination complexes. The levels of EBF1/2 can in turn be regulated through posttranscriptional degradation (EIN5 ribonuclease) and translational inhibition (EIN2 C-end). *Modified from Ju, C., Yoon, G.M., Shemansky, J.M., Lin, D.Y, Ying, Z.I., Chang, J., et al., 2012. CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 109, 19486–19491. doi:10.1073/pnas.1214848109.*

absence of the hormone, both transcription factors can be recognized by two F-box proteins, EBF1 and EBF2, and proteolyzed. When ethylene levels increase, these two F-boxes cannot only be proteolyzed themselves, but the translation of their mRNAs can also be inhibited outside the nucleus thanks to the collaboration between the EIN2 C-end and the exoribonuclease EIN5.

The accumulation of stable EIN3 and EIL1 proteins initiates a transcriptional cascade that results in the activation and repression of hundreds of genes, thus determining a coordinated response to ethylene. The *ethylene response factors* (*ERFs*), belonging to the APETALA2/ethylene response element binding protein transcription factor family, represent the most important direct targets of EIN3/EIL1 transcription factors. This is suggested not only by the number

of genes included in this superfamily (147 in the model plant *Arabidopsis*), but also by the wide range of responses (detailed in Section 5.3) that are coordinated by these elements.

The complex regulatory network of ethylene signal transduction represents not only a well evolved mechanism, but also a way to interact with many other signaling pathways, involving either other hormones or exogenous signals. A detailed description of the ethylene biosynthetic route can be found in Chang and Williams (2012) and Wang et al. (2002), while a comprehensive evaluation of the mechanisms of ethylene perception and signal transduction is given by Ju and Chang (2015).

5.2.3 Ethylene Quantification

Ethylene's story is strictly linked to the technological progress made in the methods for its quantification. Advancements in ethylene research during the 1950s and in the following decades were possible thanks to the development of gas chromatographic (GC) techniques, which currently still represent the most common tool adopted by scientists to quantify ethylene production in plants. In practical terms, whole plants or, more often, their detached organs (i.e., the fruits) are normally incubated within sealed containers of suitable volume, in order to allow the volatile gas emitted by the sample to

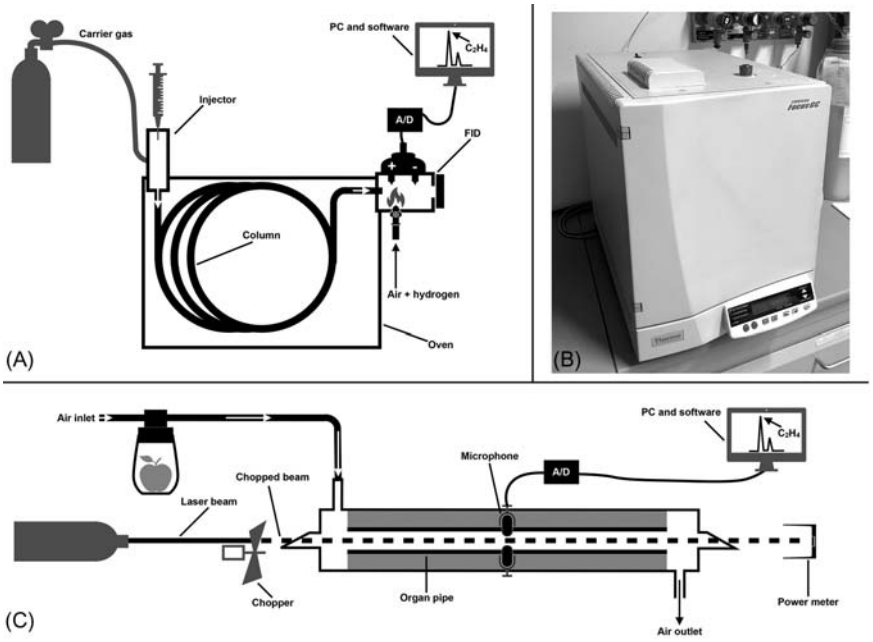

FIGURE 5.4

Methods for ethylene quantification. Schematic representation of the main elements of a gas chromatograph (GC) (A) and a common GC used in a laboratory (B). Schematic representation of the laser photoacoustic detection system (C). See text for details.

accumulate in the so-called "headspace." The headspace gas is then withdrawn with a syringe and injected into the GC.

A GC is mainly composed by three elements, whose temperature is strictly controlled to meet the quantification requirements: (1) injector; (2) column (within an oven); and (3) detector (Fig. 5.4).

The flow of the sample from the injector through the column is guaranteed by a carrier gas (i.e., air or an inert gas, such as helium). The different volatiles, among which ethylene, are separated within the column and finally elute at different times. The exact elution time of ethylene is established with a standard gas mixture of known composition. At the end of the column, ethylene is measured by a detector, usually a flame ionization detector (FID) powered by a mix of hydrogen/air. The ionization of ethylene generates an electric charge proportional to its amount, which is measured by the FID. The resulting electric signal can be then converted by suitable A/D interfaces and processed by a quantification software upon a calibration procedure.

The GC methods have allowed significant advances in ethylene research, although their detection limit is close to the ppm level. Further progress was made in the late 1980s, by adopting CO_2 laser-based photoacoustic (LPA) spectroscopy that has suitable features for the detection of trace gases in the ppb and sub-ppb concentration range. This methodology is based on the generation and detection of pressure waves (i.e., sound) inside a resonant cell, where the gas samples are placed or allowed to flow through. Samples are exposed to a modulated radiation, which can be absorbed at specific wavelengths ($\sim 10\,\mu m$ for ethylene), heat the sample and, thus, generate a sound signal. This sound is measured by highly sensitive microphones, inside the cell, and converted into an electric signal, which is filtered and detected by an amplifier (Fig. 5.4).

More recently, the light-emitting diode (LED) technology has allowed setting up of portable LPA devices that can be used even for measurements of ethylene production directly "in the field."

The significant evolution and current developments of ethylene measurement approaches in recent years are described in depth by Caprioli and Quercia (2014).

5.3 ETHYLENE PHYSIOLOGY AND CROSSTALK WITH OTHER HORMONES DURING POSTHARVEST

Ethylene is widely considered the dominating hormone responsible for the activation and acceleration of climacteric fruit ripening and plant senescence. Sound evidence, based on chemical and biotechnological manipulation experiments, has unequivocally proven the role of ethylene in determining the onset and progression of ripening in climacteric fruits, in inducing certain ripening-associated events in nonclimacteric fruits, as well as in accelerating overall senescence in plant tissues (reviewed by Seymour et al., 2013). The

action of ethylene is amplified following abiotic (mechanical damage, wounding, wilting, etc.) or biotic (attack by pathogens) stress conditions. Counteracting ethylene action and senescence is a central issue in the context of extending the shelf-life of commodities.

Ethylene action in pre- and postharvest ripening and senescence underlies the development of both positive and negative attributes, such as the loss of firmness, the synthesis of pigments and of aroma volatiles, as well as the stimulation of ethylene biosynthesis itself. All these events are enhanced by treatments with exogenous ethylene and are delayed or blocked by inhibiting ethylene perception or biosynthesis either chemically (e.g., by treatments by 1-MCP or silver ions, or AVG, respectively) or genetically, providing compelling evidence on the master regulatory role played by this hormone. Ethylene-induced loss of firmness is a primary aspect in postharvest life influencing decay and susceptibility to mechanical stress and pathogen infection. It is brought about by means of transcriptional activation of several genes encoding enzymes involved in cell wall remodeling (e.g., glucanases, EGAs; expansin, EXP; beta-galactosidase, GAL; pectin methylesterase, PME; pectate lyase, PL; polygalacturonase, PG; xyloglucan endotransglucosidase/hydrolases, XET) and of the regulatory factors (i.e. a Rab GTPase encoding gene) responsible for the correct secretion of cell wall enzymes to the apoplastic space. Ethylene also controls color change, through carotenoid biosynthesis (e.g., by enhanced transcription of phytoene synthase, PSY) and chlorophyll degradation, by inducing the overexpression of chlorophyllase encoding genes. The process of chlorophyll degradation, in particular, is responsible for the "degreening" process of florets (e.g., broccoli), fruits (e.g., citrus and bananas), and leafy vegetables (e.g., spinach, etc.), occurring during postharvest storage and representing a visible landmark of senescence.

The regulation of these events by ethylene is the final outcome of the interaction with a number of factors, among which plant growth regulators represent a key element. The concept that ethylene does not exert its ripening- and senescence-inductive action alone, but rather in the context of a finely tuned and balanced interplay with other hormones, has been put forward as soon as ethylene's implication in these processes was postulated. This hypothesis has progressively gained biochemical and, more recently, transcriptomic, proteomic, and biotechnological evidences in support (reviewed by Iqbal et al., 2017). Overall, considering the traditional categories of plant hormones, gibberellins (GAs), auxins (IAAs), and cytokinins (CKs) seem to exert in general an antagonistic role against ethylene's action, while abscisic acid (ABA) plays a synergistic role (Fig. 5.5).

There are common as well as divergent specific effects brought about by each hormonal category. However, a prudent approach must be always adopted before drawing general conclusions, since it has to be considered that, in some cases, opposite responses can be triggered by the same growth regulator depending on the concentration, the timing, and the mode of application and the developmental and physiological state of the plant produce under investigation.

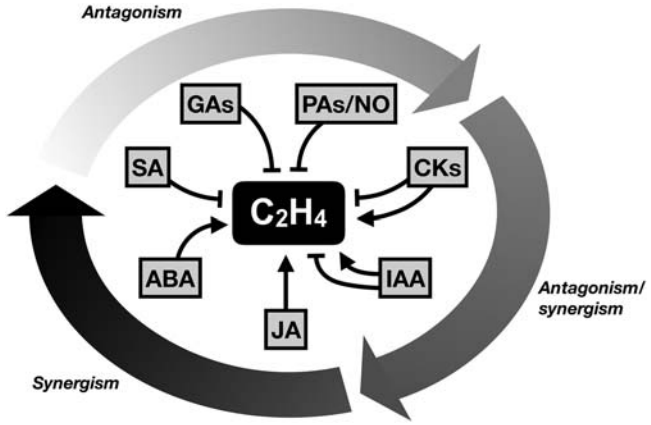

FIGURE 5.5

Synergistic and antagonistic relationships between ethylene and other hormones. Abscisic and jasmonic acid (ABA and JA) play a synergistic role together with ethylene in promoting ripening and senescence of fruits and vegetables, respectively. Nitric oxide (NO), poly-amines (PAs), gibberellins (GAs), and salicylic acid (SA) play a general antagonistic role. Auxins (IAAs) and cytokinins (CKs) may exert either antagonistic or synergistic actions, depending on the fruit/vegetable developmental stage (see text for details).

Auxins undergo a decrease in terms of free active levels, along with a corresponding increase in their inactive conjugated forms, before the onset and during ripening in both climacteric and nonclimacteric fruits as well as during senescence, supporting their antagonistic role with ethylene. The preharvest exogenous application of auxins (such as the synthetic auxins NAA or 2,4D) on immature fruits normally results in a delay in fruit ripening and senescence (e.g., in strawberries, grape, banana, tomato), manifested by a slower loss of firmness and chlorophyll content during postharvest, a slower accumulation of pigments, and delayed overall postharvest decay. Conversely, when sprayed after the onset of ripening, auxins may induce the enhancement of ripening in some systems, mostly climacteric fruits (peach, nectarine, and apple), through the stimulation of transcription of ACS-encoding genes and therefore of ethylene biosynthesis. An ethylene-counteractive role, similar to that played by auxins, can be also attributed to CKs, which are well known for their preventive role against yellowing of leaves in several plant species. Both pre- and postharvest exogenous applications of benzyl adenine (BA), zeatin, or forchlorfenuron (CPPU) result in the inhibition of ethylene-dependent chlorophyll degradation, a consequent delay in postharvest "degreening," loss of firmness, and overall decay, through the downregulation of chlorophyllase and cell wall enzymes, in both climacteric and nonclimacteric (e.g., strawberry, citrus) fruits as well as in broccoli florets and leafy vegetables. As for auxins, the preripening application of cytokinins reduces ethylene biosynthesis and sensitivity and the respiration climacteric rise while the application after the onset of ripening may result in an opposite effect. Even though these general conclusions can be considered overall valid, there are reports in the literature showing opposite effects. For example, in kiwifruit early (after bloom) preharvest treatments

with CK enhanced starch accumulation, followed by faster ripening. Treatments of broccoli florets with CKs lead to a decreased climacteric respiration, a delayed overall yellowing process, and longer shelf-life, while increasing levels of ethylene evolution, thus suggesting that some effects mediated by plant growth regulators may be evoked through differential effects on specific ripening-associated events. A similar picture can be drawn for GAs, also reported to counteract in some fruits the loss of firmness and the consequent spoilage due to pathogen attack during postharvest storage, and a stronger synergistic effect may occur when GAs and CKs are used in combination to delay chlorophyll breakdown and prolong shelf-life. Indeed, all these effects seem most frequently to involve the downregulation of ethylene biosynthesis and sensitivity and are counteracted by supplying exogenous ethylene, suggesting that all these growth regulators act in the context of a reciprocal balance coordinating their biosynthesis, metabolism, and perception/action. It must also be noted that use of early (at bloom) sprays with CKs + GAs to stimulate fruit development also results in some cases in fruit with longer shelf-life and delayed senescence. However, in this latter case, the antagonistic effect toward ethylene probably takes place through indirect crosstalk mediated by changes in metabolism, for example, manifested by an increased uptake of nutrients such as Ca^{2+} or sugars. An important antisenescence action, in clear antagonism with ethylene, is also played by new emerging growth regulators, among which polyamines (PAs) deserve mentioning. PAs cannot be regarded as bona fide hormones but can be nevertheless considered as general plant growth regulators. PAs (spermine, spermidine, and putrescine) display high levels in young tissues and progressively decrease in ripening fruits, reaching low levels in all senescent plant tissues. The use of pre- and postharvest PA sprays has been consistently and repeatedly shown to downregulate ethylene production and responses in a number of systems, enhancing fruit firmness by suppressing cell wall enzymes. Evidence is accumulating in support of a similar role for salycilic acid (SA), even though for SA action the data are scanty and, in some cases, apparently contradictory. Finally, among the small molecules with an ethylene antagonistic role, sound evidence is available for nitric oxide (NO), which downregulates ethylene biosynthesis by inhibiting the transcription and activity of its biosynthetic enzyme ACS and ACO through protein S-nitrosylation.

ABA can be considered a well-established ethylene allied in promoting ripening and senescence. ABA levels and transcript of genes involved in its biosynthesis (e.g., 9-cis-epoxycarotenoid dioxygenase) increase before ripening (and before the ethylene climacteric in climacteric fruits) in nonclimacteric (e.g., grape, strawberry) and climacteric (e.g., tomato) fruits. The inhibition of ABA biosynthesis or action, either chemically or through biotechnological manipulation, has been shown to result in a delay of the respiratory and ethylene climacteric, reduction of transcription of cell wall enzymes encoding genes, and lower accumulation of pigments, while exogenous ABA applications result in enhanced ethylene biosynthesis. Overall, these data clearly support a role for

ABA in the promotion of fruit ripening as well as of postharvest senescence, at least in some cases by means of a stimulatory effect on ethylene production. Similar experiments on climacteric (tomato) and nonclimacteric (grape) fruits have suggested a role consistent with that of ABA for brassinosteroids (BRs) and for jasmonic acid (JA), even though evidence in support is still limited. As a concluding remark, the progressive discovery of new elements in the cross-talk with ethylene in controlling ripening and senescence opens up novel opportunities for intervention in the context of improving the shelf-life of products. The new tools that may be developed will need to be increasingly eco-friendly and, besides the development of chemicals with low or no toxicity that can interfere with ethylene's action, such as the promising use of PAs, may include new molecular targets that could be tackled to interfere with ethylene-dependent responses through last-generation biotechnological and genetic approaches.

5.4 THE IMPACT OF POSTHARVEST CONDITIONS ON ETHYLENE PHYSIOLOGY

After harvest, horticultural commodities undergo several changes in terms of physicochemical properties, metabolism, and composition, leading to a more or less rapid evolution of ripening and senescence, as well as an increased susceptibility to postharvest pathogens, resulting in a reduced commercial- and shelf-life. Physical stress (wounding, bruising) and microbial infection not only cause direct losses, but also induce physiological responses, such as an increase in ethylene biosynthesis leading to an acceleration of ripening and of the onset of senescence, that can extend also to the surrounding commodities. Control of these factors, which can be obtained mostly by preventing mechanical damage, is a prerequisite for a successful storage. After detachment from the mother plant, fresh fruits and vegetables are still alive and, due to the high water content, are metabolically active and react to different stimuli present in the environment. Since high metabolism results in high rates of deterioration and shelf-life shortening, several strategies and techniques can be applied in order to slow down or delay ripening/senescence-related metabolic processes. The main goal of the storage protocols is to affect and control the following factors: (1) respiration; (2) biosynthesis and action of ethylene; (3) changes in composition and structure; (4) water loss (transpiration); (5) physiological disorders; and (6) pathogen infections.

Even though the goals of postharvest techniques may vary in relation to the crop and the final destination of the produce, a common feature is the need for reducing activities and processes related to primary metabolism. In addition to respiration, specific protocols or conditions applied after harvest are also effective, directly or indirectly, in altering ethylene physiology with marked effects on the ethylene-dependent processes typically characterizing the ripening of the climacteric fruit (see Section 5.3) and the senescence of

organs such as leaf vegetables with significant commercial benefits through the extension of shelf-life and the reduction of postharvest wastage.

Several postharvest treatments can be applied in order to delay ripening and senescence, some of which are innovative and still under evaluation of their efficacy. The most commonly applied storage techniques are: (1) low temperature; (2) controlled (CA) and/or modified atmosphere (MA), and (3) ethylene antagonists. CA and MA, as well as the application of ethylene antagonists, are used in combination with low-temperature regimes, so effects are additive.

5.4.1 Temperature

Lowering the temperature of produce soon after harvest and maintaining the cold chain throughout the postharvest phase is considered the main pillar of storage technology for appropriate handling of horticultural produce. According to Adel Kader, "*temperature management is the most effective tool for extending the shelf-life of fresh horticultural commodities*." The optimal temperature for storage varies according to the produce (origin, plant organ, developmental stage, etc.) and must be applied according to these two main principles: (1) low temperatures slow down metabolism and decrease the rate of compositional changes; and (2) temperatures below a specific threshold (different in relation to the fruit produce) and/or a prolonged cold storage induce chilling injuries. Refrigeration has a profound impact on the overall metabolism of plants and plant organs. In order to measure the changes for a biological system as affected by temperature, the Q_{10} temperature coefficient is used. Q_{10} indicates the rate of reactions in a biological system as a result of a temperature increase of 10°C. For most crops, within a temperature range of 5°C–25°C, Q_{10} values associated with respiration are around 2.0–2.5. This means that by lowering temperature from 15°C to 5°C the respiration is decreased by a factor of 2, with obvious benefits in terms of shelf-life, considering that the respiration rate of fresh fruits and vegetables is often used as a predictor of the effect of temperature on the overall metabolism.

In addition to respiration, low temperature applied in storage facilities markedly affected ethylene physiology. Since the discovery of ethylene as a natural compound produced by plant tissues and organs and, later, the biochemical and molecular characterization of the main steps of the hormone biosynthesis, the effects of cold storage on ethylene production of harvested hort produce have been described, in particular regarding the postharvest behavior of climacteric fruit. A steady decrease in the rate of ethylene biosynthesis is observed by lowering temperatures. This is the result of a downregulation of ACS and ACO gene expression as observed in both tropical (e.g., banana) and temperate (e.g., apple, peach) fruit species. In tomato fruit stored at 3°C, alteration of ethylene production correlates with the altered expression of specific ACC synthase (*ACS2*, *ACS4*) and ACC oxidase (*ACO1*) genes, involved in the onset of the climacteric rise. In addition to gene expression, low temperatures are also effective in altering enzyme activities: ACS activity

in particular shows a sigmoidal pattern in a range of temperature between 5°C and 35°C. Also, ethylene perception and the signal transduction pathway is affected by cold storage. In tomato fruit the expression of the receptor genes *LeETR1*, *NR*, *LeETR4*, and that of the signal cascade, *LeCTR1*, *LeEIL3*, *LeEIL4*, and *LeERF3*, is altered by chilling. Interestingly, also in nonclimacteric fruit (e.g., grapefruit) the expression of ethylene receptor genes is affected by low-temperature storage and ethylene appears to be implicated in the transcriptional regulation of ERFs under cold storage.

Storing fruit under cold conditions may result in the onset of chilling injuries (CIs). For example, in tomatoes, prolonged exposure to low temperature (about 7−13°C) induces the appearance of injury symptoms such as aroma loss, blotchy ripening, excessive softening, pitting, susceptibility to decay, electrolyte leakage, and failure to ripen. In peaches, internal breakdown, flesh browning, and bleeding and mealiness are associated with storage in the range of 2−8°C. The role of ethylene in the onset and development of chilling injuries is controversial and appears to be variable in different species. In many cold-sensitive fruits, low temperature stimulates ethylene production and this is considered as one of the inducing factors leading to the onset of CIs. This has been confirmed by the fact that in fruit with suppressed ethylene production (transgenic lines) or treated with ethylene antagonists (e.g., 1-MCP, see below), CIs are reduced. However, in some other fruits (e.g., peaches) the development of CIs may be due to the reduction or inhibition of ethylene production and strategies applied to increase ethylene production before or during cold storage are effective in lowering the incidence of CIs.

An interesting aspect concerning storage of fruits at low temperature is that in some cultivars of apples, pears, and kiwifruit, ethylene production is stimulated after more or less prolonged chilling exposure, when fruits are moved to room temperature (20°C). In these fruits, rapid ripening- associated processes, such as loss of flesh firmness, are coupled with higher levels of ethylene production, as a result of an upregulation of both ACS and ACO gene expression and enzyme activities. If for some fruit species this physiological behavior has a positive impact on quality (without chilling winter pears do not develop the best eating quality during shelf-life), for some other crops it represents a negative aspect that could be prevented by storing fruit in a controlled atmosphere.

5.4.2 Atmosphere Composition

In addition to temperature, atmosphere composition plays a key role in affecting the postharvest life of fruit produce: this is in particular related to the concentrations of oxygen, carbon dioxide, and ethylene. The control of these parameters represents the basis of the storage technique called controlled atmosphere (CA). In addition to CA, an expensive system widely applied only for specific fruits such as apples, winter pears, kiwifruits, and a few others, is MA and in particular modified atmosphere packaging (MAP), which represents an appropriate solution for prolonging the storage and shelf-life of different

hort commodities. Differently from CA, where gas concentrations are strictly monitored and controlled, in MA and MAP the gas balance is achieved by the respiratory activity of the product.

The reduction of oxygen level and the increase in carbon dioxide concentration (associated with low temperature) lead to a reduction in respiration as well as ethylene biosynthesis and action, and subsequently to better maintenance of commercial-quality parameters of the produce. It has been recognized that ACS is the major site at which elevated CO_2 and reduced O_2 atmospheres inhibit C_2H_4 biosynthesis in ripening apple and peach fruit. Also, the conversion of ACC to ethylene is affected, as observed in pears where changes in ACO mRNA levels and protein accumulation occur during CA storage conditions. The extremely low oxygen concentrations applied in the advanced CA protocols (e.g., initial low oxygen stress (ILOS) and dynamic controlled atmosphere (DCA)) have a pronounced effect on the overall metabolism and reduce the expression of a number of genes including those responsible for the ethylene biosynthesis as recently observed in Granny Smith apples stored at 0.4 and 0.8 kPa oxygen. In addition to the biosynthetic pathway, elements of the signal transduction pathways are also affected by hypoxia. As observed in model species (*Arabidopsis*), ethylene-responsive factors (ERFs) seem to be involved in oxygen-sensing mechanisms also in apple fruit tissues.

The inhibition of ethylene and ethylene-dependent processes (including autocatalytic synthesis) by CO_2 has been associated with the competition of the gas with ethylene at the receptor-binding site. Reduced ACS mRNA accumulation occurs in tomato and peach fruit exposed to high CO_2 levels while ACO members appear to respond differently to such conditions. The mechanisms by which CO_2 alters ethylene physiology are still to be fully discovered.

A key factor that has to be considered in storage facilities is the elimination of the ethylene produced by commodities and product. Some fruit species (e.g., kiwifruit) are extremely sensitive to even very low ethylene concentrations in the atmosphere. The use of adequate ventilation and ethylene removal systems (through chemical absorbers or catalytic conversion) is a requirement for a successful prolonged storage. For MAP, the removal of undesirable ethylene greatly improves the benefits of this technique and adsorbers (activated carbons) or oxidizers (potassium permanganate) are widely used in the so-called active packaging technology (see also Section 5.4.3).

5.4.3 Other Postharvest Treatments

In addition or as an alternative to refrigeration with or without CA/MA, other physical, chemical, and biological treatments may be applied to maintain fresh-like quality, preserve the nutritional/nutraceutical value and meet the safety standards of fruits. Heat treatments have been proposed as an alternative to chemical treatments to control fruit decay and/or for killing insects (pesticide quarantine treatment) but also for reducing the impact of CIs during the following refrigerated storage. In general, heat treatments are of

short duration (from a few seconds to several minutes/hours) and performed in hot water, hot air, or vapor heat at temperatures in the range of 40°C–55°C, depending on the commodity and the method. In general, heat treatments induce a delay of ripening in climacteric fruit due a reduction of ethylene production as ACS and ACO are among the enzymes affected by the treatment. However, depending on the treatment parameters (temperature and time), opposite effects can also be observed: in tomato fruit, for example, hot-water treatments performed before refrigerated storage may induce CI tolerance, and this effect seems to be related to the restoration of ethylene biosynthesis and signaling.

With the aim of controlling pathogens and reducing postharvest decay, advanced technologies such as edible films or coatings and antimicrobial packaging are increasingly used. Edible films or coatings are thin layers of material from different sources (natural, synthetic) applied to the surface of fresh produce providing an additional natural barrier. This reduces transpiration and, acting as a gas barrier, a modified atmosphere is established around the fruit surface with similar effects as those described in Section 5.4.2. In antimicrobial packaging, active ingredients are added to the packing system/material, resulting in the prevention of microbial growth. Some natural compounds, such as essential oils, are used in the substitution of chemical substances in general not permitted for edible produce. Recent advancements incorporate edible coating or nanoemulsions with essential oils and, besides controlling spoilage, these technical solutions result in altering ripening physiology with a decrease in respiration and ethylene production as observed in both whole fruit and fresh-cut produce.

Ultraviolet light, and in particular UV-C (far-UV) at low doses, is effective in inducing resistance against pathogens in harvest produce and is also effective for surface decontamination in fresh-cut produce. In addition, the application of UV light delays tomato fruit ripening and this effect seems to be due to the activation of ERFs that could act as regulators of metabolic pathways during ripening.

5.4.4 Specific Inhibitors of Ethylene Biosynthesis and Perception

Specific inhibitors of ethylene biosynthesis or antagonists of its action can also be effective in improving the storage performance of horticultural crops. For edible produce, the use of some of them is impossible, difficult, too expensive, or forbidden by law as, in the EU, for almost all postharvest chemicals on fruit and vegetables. This is the case of the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG), known commercially as ReTain, effective in inhibiting ACS enzymatic activity and, as a consequence, reducing ethylene production. Only preharvest applications of ReTain are allowed and performed in the fruit industry, in particular on pears, apples, and peaches, leading in general to a delay of postharvest ripening due to reduced production of

ethylene. Preharvest ReTain applications on apples are also effective in limiting losses due to the reduced mature fruit drop. Considering the inhibitors of ethylene action, since the early 1970s silver thiosulfate (STS) has been widely used to enhance the longevity of flowers and display life of potted plants that are ethylene-sensitive. However, for its toxicity, STS cannot be used on edible fruits and vegetables. A great advancement in optimizing the storage protocols of several horticultural crops has been represented by the discovery by Eduard Sisler of 1-methylcyclopropene (1-MCP), a cyclic olefin extremely effective in competing with ethylene at the level of receptors. This compound, now successfully used worldwide for ethylene-sensitive horticulture produce, including edible and ornamental crops, is commercially applied as a stable powder that easily releases a gas when dissolved in water. It is effective at very low concentrations (0.1−1 μL/L) and for short exposures (a few hours can be sufficient) and, as a gas, it can be easily used in storage rooms. The main effects of 1-MCP treatments are those of reducing ethylene production and respiration rate, thus controlling the quality parameters dependent on ethylene (loss of firmness, in particular). Several apple varieties benefit from the application of 1-MCP, which can be effective also on other fruit produce (pears, bananas, plums, tomatoes, etc.). Together with these positive effects (prolonging the commercial life), some negative effects result from treatment with the ethylene antagonist. This is the case for some apple varieties that do not fully develop the typical aroma. A huge scientific and technical literature demonstrates that 1-MCP effects are variable in relation to the fruit species and variety and several factors (concentration, duration, and temperature of the treatment, developmental stage of the fruit, posttreatment storage conditions) may affect the final result of the application. Even though the effects of 1-MCP treatments are markedly present in climacteric fruit, under specific conditions positive effects of 1-MCP can also be observed for maintaining quality and/or affecting some physiological processes in nonclimacteric fruit.

5.5 BIOTECHNOLOGICAL APPROACHES

The ever-increasing identification of the molecular factors recruited by ethylene during postharvest ripening and senescence has provided a number of possible targets for biotechnological interventions to enhance the shelf-life of fruits and vegetables. Historically, biotechnological approaches have followed the progressive discovery of the main players of the "ethylene scene." The first attempts to hasten shelf-life were mostly aimed at controlling specific ethylene-dependent responses, among which enhancing firmness maintenance in fruits through the manipulation of genes encoding cell wall enzymes clearly appeared as one of the most important targets to improve postharvest storage life. The biotechnological downregulation of expression of cell wall encoding genes either by RNA antisense technology and, more recently, by RNA interference (RNAi), proved to be at least in part successful in conferring better maintenance of fruit firmness for a certain number of enzymes, among which PGs, PLs, and PMEs should be listed, while it did not do so for other enzymes such

as, for example, EGases. Examples of successful manipulation of PLs and PMEs, resulting in an increased shelf-life and subsequent reduced postharvest spoilage, are available for both climacteric (tomato) and nonclimacteric (strawberry) fruit models. It must be noted that the lack of effects of several cases of manipulations of cell wall enzymes encoding genes could be ascribed to the fact that cell wall rearrangements taking place during ripening and influencing postharvest storability are the result of complex sequentially coordinated action of several enzymes. This in many cases can make the manipulation of single enzymes overall ineffective on fruit firmness.

As far as climacteric fruits are concerned, soon after the identification of the genes encoding the enzymes involved in the last two steps of ethylene biosynthesis (ACS and ACO), a logical further development for biotechnological control of postharvest life was the application of gene-silencing techniques for the reduction of endogenous ethylene release. This approach moved the target to an upstream level, where the rationale was counteracting the action of the master hormonal regulator of ripening and senescence, thus delaying all downstream events. Tomatoes transformed with antisense ACS and ACO genes, obtained through pioneering studies, have provided the proof of concept and paved the way to obtain fruits with very low ethylene biosynthesis and significantly delayed overall ripening and senescence. Since then, the same approach has been applied with success on a number of climacteric fruits, ranging from melon to apple, as well as on flowers (e.g., carnation) and florets (e.g., broccoli) with evident inhibitory effects on postharvest senescence and with increased shelf-life. Importantly, the biotechnological control of ethylene biosynthesis can be overcome by exogenous application of ethylene, thus giving the opportunity of removing the endogenous block and re-establishing normal ripening when required. Additional approaches downregulating ethylene biosynthesis have exploited the use of transgenes responsible for decreased levels of the ethylene precursors SAM and ACC through their enhanced metabolism. Such applications have included the overexpression of bacteriophage T3 SAM hydrolase and bacterial ACC deaminase encoding genes and resulted in overall lower endogenous ethylene release and longer shelf-life. Also, the overexpression of SAM decarboxylase, controlling the diversion of decarboxylated SAM toward the biosynthesis of PAs, has been successfully used for improving tomato fruit firmness, quality, and postharvest life, while unexpectedly leading to increased ethylene evolution, possibly through interference exerted by PAs on ethylene signaling.

After the discovery of the molecular components of ethylene perception and signaling, further biotechnological developments have included the exploitation of such elements in a number of systems. Due to the nature of their mode of action as negative regulators of ethylene responses, the overexpression of mutated versions of the ethylene receptors (e.g., the *Arabidopsis etr1-1* or the mutated receptor of the *Never Ripe* tomato mutant, *Nr*) has been used to confer dominant insensitivity to the hormone, thus making the transformed fruit or plant completely or partially insensitive to ethylene in a constitutive

manner. This approach leads to an intrinsic inability of the plant to respond to endogenous ethylene but also to exogenously applied ethylene. A consequence of such a constitutive ethylene insensitivity is definitely a rather significant increase in shelf-life and much slower decay. However, this also implies the impossibility of restoring a normal ripening process by providing exogenous ethylene and therefore of obtaining fruits of acceptable quality for the market. Similar nearly overlapping results, with the same pros and cons, have been obtained by inhibiting the expression of ERF and EIN encoding genes, thus acting on components of the signaling pathway downstream of ethylene perception, by antisense approaches. Biotechnological control of ethylene biosynthesis and signaling has proven to be of interest for the improvement of the overall postharvest quality of fruits and vegetables, however future studies should be devoted to set suitable tools for the conditional control of such transgenes in order to make it possible to re-establish a normal fruit-ripening syndrome when desired. Also, new approaches besides the use of antisense, cosuppression, or RNAi-mediated silencing technologies, may be exploited for improved efficiency and better spatiotemporal control and may include the use of artificial miRNAs and CRISPR/Cas9 techniques to open new opportunities for highly targeted gene manipulations.

REFERENCES

Caprioli, F., Quercia, L., 2014. Ethylene detection methods in post-harvest technology: a review. Sens. Actuat. 203 (2014), 187–196. Available from: https://doi.org/10.1016/j.snb.2014.06.109.

Chang, C., Williams, M.E., 2012. Ethylene. Teaching tools in plant biology: lecture notes. Plant Cell (Online) . Available from: https://doi.org/10.1105/tpc.110.tt1010.

Iqbal, N., Khan, N., Ferrante, A., Trivellini, A., Francini, A., Khan, M., 2017. Ethylene role in plant growth, development and senescence: interaction with other phytohormones. Front. Plant Sci. 8, 475. Available from: https://doi.org/10.3389/fpls.2017.00475.

Ju, C., Chang, C., 2015. Mechanistic insights in ethylene perception and signal transduction. Plant Physiol. 169 (1), 85–95. Available from: https://doi.org/10.1104/pp.15.00845.

Ju, C., Yoon, G.M., Shemansky, J.M., Lin, D.Y., Ying, Z.I., Chang, J., et al., 2012. CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A. 109, 19486–19491. Available from: https://doi.org/10.1073/pnas.1214848109.

Seymour, G.B., Chapman, N.H., Chew, B.L., Rose, J.K., 2013. Regulation of ripening and opportunities for control in tomato and other fruits. Plant Biotechnol. J. 11, 269–278. Available from: https://doi.org/10.1111/j.1467-7652.2012.00738.x.

Wang, K. L.-C., Li, H., Ecker, J.R., 2002. Ethylene biosynthesis and signaling networks. Plant Cell 14 (Suppl), s131–s151. Available from: https://doi.org/10.1105/tpc.001768.

FURTHER READING

Bakshi, A., Shemansky, J.M., Chang, C., Binder, B., 2015. History of research on the plant hormone ethylene. J. Plant Growth Regul. 34, 809–827. Available from: https://doi.org/10.1007/s00344-015-9522-9.