Biosynthesis of Carotenoid-Derived Plant Signaling Molecules

Dissertation by

Lina Baz

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EXAMINATION COMMITTEE

The thesis of Lina Baz is approved by the examination committee.

Committee Chairperson: Prof. Salim Al-Babili

Committee Members: Prof. Stefan Arold, Prof. Mark Tester, Prof. Mmatias Zurbriggen
ABSTRACT

Biosynthesis of Carotenoid-Derived Plant Signaling Molecules

Lina Baz

Carotenoids are precursors of hormones and signaling molecules across all kingdoms of life. An increasing body of evidence suggests the presence of yet unidentified carotenoid-derived metabolites (apocarotenoids) with developmental and regulatory functions, besides the known plant hormones abscisic acid (ABA) and strigolactones (SLs). Generally, apocarotenoid synthesis is initiated by carotenoid cleavage dioxygenases (CCDs), which constitute a ubiquitous family of non-heme iron enzymes. In SL biosynthesis, an iron-binding cis/trans-isomerase, DWARF 27 (D27) converts all-trans-β-carotene into 9-cis-β-carotene. This reaction is followed by a double bond cleavage at 9, 10 position, mediated by the stereospecific CCD7. The cis-configured cleavage product of CCD7, 9-cis-β-apo-10'-carotenal, is simultaneously cleaved, triple-oxygenated and rearranged by CCD8, to produce carlactone (CL). CL is a central metabolite and the precursor of a wide range of SLs.

The aim of this work is to investigate whether CCD8 synthesize CL-like compounds from other 9-cis-configured apocarotenoids to confirm their presence and synthesis in planta. We showed that CCD8 enzymes from different plants produce a hydroxylated carlactone (3-H-CL) from 9-cis-3-OH-β-apo-10'-carotenal in vitro. In addition, we detected 3-H-CL in Nicotiana benthamiana leaves transiently expressing the CL biosynthesis enzymes from rice and Arabidopsis. 3-H-CL is biologically active, as shown by Striga hermonthica seed germination assay and by its effect on the high-tillering phenotype of the rice d10 mutant. We also confirmed that 3-H-CL is a natural metabolite by detecting it in roots of the rice SL perception mutant d14.

In a second project, we investigated the activity of three rice CCDs in vitro and showed that one of them (zaxinone synthase; ZAS) is an apocarotenoid cleavage enzyme with a clear preference for the substrate all-trans-3-OH-β-apo-10'-carotenal, as suggested by a kinetic study. ZAS produces two products, the C18 ketone zaxinone and an unstable C9 dialdehyde that could be identified by LC-MS after derivatization. Activity tests were performed with crude lysates of overexpressing Escherichia coli cells and with purified enzyme. We established that zaxinone is a natural metabolite present in planta. Investigations of a corresponding rice mutant (zas) and activity bioassays performed by our group demonstrate that zaxinone a novel signaling molecule required for normal rice growth and development.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>AM</td>
<td>arbuscular mycorrhizal</td>
</tr>
<tr>
<td>AU</td>
<td>arbitrary unit</td>
</tr>
<tr>
<td>At</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>B-HYD/ BCH</td>
<td>β-carotene hydroxylase</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CCD</td>
<td>carotenoid cleavage dioxygenase</td>
</tr>
<tr>
<td>CCO</td>
<td>carotenoid cleavage oxygenases</td>
</tr>
<tr>
<td>CL</td>
<td>carlactone</td>
</tr>
<tr>
<td>CLA</td>
<td>carlactonoic acid</td>
</tr>
<tr>
<td>CrtI</td>
<td>bacterial carotene desaturase</td>
</tr>
<tr>
<td>CRTISO</td>
<td>carotene-cis-trans-isomerase</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DAD</td>
<td>decreased apical dominance</td>
</tr>
<tr>
<td>DMAPP</td>
<td>dimethylallyl diphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ε-LCY</td>
<td>ε-lycopene-cyclase</td>
</tr>
</tbody>
</table>
Fe ferrous iron ($Fe^{2+}$)
5-DS 5-deoxystrigol
GST glutathione S-transferase
HPLC High Performance Liquid Chromatography
IAC The Instituto Agronomico de Campinas
kDa kilo Dalton
LBO Lateral Branching Oxidoreductase
LC-MS Liquid Chromatography Mass Spectrometry
M molar
mAU milli arbitrary unit
MeCLA methyl carlactonoate
min minutes
MAX more axillary growth
MEP 2-C-methyl-D-erythritol-4-phosphate pathway
NADPH Nicotinamide adenine dinucleotide phosphate H
NCED nine-cis-epoxycarotenoid dioxygenase
NMR Nuclear magnetic resonance
Ps Pisum sativum
RMS ramosus
RT room temperature
Sl Solanum lycopersicum
SDS sodium dodecyl sulfate
SL strigolactone
SPME  Solid phase micro extraction

SynACO  *Synechocystis* apocarotenoid cleavage oxygenase

TBME  *tert*-butylmethylether

TBSV  Tomato Bushy Stunt Virus

v/v  volume per volume

wt  wild-type

μg / μl / μM  microgram / microliter / micromolar

ZAS  zaxinone synthase
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1. Introduction

1.1. Carotenoids

Carotenoids are isoprenoid compounds generally consisting of \( C_{40} \) long hydrocarbon chains with a conjugated double-bond system. The number and stereo-configuration of double bonds in carotenoid backbone determine their light absorption capacity and, hence, their colors. Although they serve a variety of important functions in all living organisms, carotenoids are produced only by photosynthetic, and some heterotrophic microorganisms (Walter and Strack, 2011). Carotenoids are essential for the photosynthesis process, as they enhance the efficiency of light harvesting and protect chlorophylls and other important cellular components from photo-oxidation (Hashimoto et al., 2016). Colors of accumulated carotenoids in many flowers and fruits promote the communication between plants and animals by attracting pollinators and distributors (DellaPenna and Pogson, 2006). Animals need carotenoids in their diet for their crucial role as antioxidants and precursors of the vision chromophore retinal and vitamin A (retinol). In addition to retinoids, carotenoids serve as a precursor of many compounds, generally called apocarotenoids, which comprise a family of important multifunctional metabolites. According to their structure, carotenoids are divided into two groups; carotenes, the oxygen-free hydrocarbons, such as \( \beta \)-carotene, and their oxygenated derivatives, xanthophylls, such as lutein and zeaxanthin. Carotenoids can also be sub-classified by their end groups in to acyclic, monocyclic and bicyclic, or by the stereo-configuration of their double bonds (\( cis/trans \)) (Floss et al., 2008; Jia et al., 2017; Walter and Strack, 2011).

The first step in carotenoid biosynthesis (Figure 1) is the condensation of two molecules of geranylgeranyl diphosphate (\( C_{20} \)) to form 15-\( cis \)-phytoene (\( C_{40} \)). This reaction is mediated by the enzyme Phytoene synthase. In the following steps, the number of conjugated double bonds in
phytoene is increased by a series of desaturation and cis/trans-isomerization reactions to yield all-trans-lycopene. This part of the pathway is known as the poly-cis pathway and is catalyzed by the enzymes Phytoene synthase (PSY), Phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), and at least two cis/trans isomerases, i.e. Carotene isomerase (CrtISO) and ζ-carotene isomerase. Subsequently, two β-ionone rings are introduced to the all-trans-lycopene by the action of Lycopene-β-cyclase (LCY-β), leading to all-trans-β-carotene that marks the β-branch of the pathway. The conversion of all-trans-lycopene by the Lycopene-ε-cyclase (LCY-ε) leads to all-trans-α-carotene, the first molecule in the second branch of the pathway. Hydroxylation of the two ionone rings in α-carotene leads to all-trans-lutein, while hydroxylation of the two rings of β-carotene at the same positions produces all-trans-zeaxanthin. Epoxidation of the β-ionone rings in all-trans-zeaxanthin gives rise to all-trans-violaxanthin that is converted to all-trans-neoxanthin, the final product of the β-branch (DellaPenna and Pogson, 2006; Jia et al., 2017; Moise et al., 2014).
Figure 1. Carotenoid biosynthesis in plants. The first step is catalyzed by Phytoene synthase (PSY) that mediates the condensation of two geranylgeranyl diphosphate (GGPP; C\textsubscript{20}) molecules into the C\textsubscript{40} product 15-cis-phytoene. Next, Phytoene desaturase (PDS) introduces two double bonds at C11 and C11′, accompanied by trans to cis isomerization at C9 and C9′. ζ-carotene isomerase (Z-ISO) converts 9,15,9′-tri-cis-ζ-carotene into 9,9′-di-cis-ζ-carotene, the substrate of ζ-carotene desaturase (ZDS) which introduces two double bonds at C7 and C7′ to produce 7,7′,9,9′-tetra-cis-lycopene. Carotene isomerase CrtISO catalyzes the isomerization of the four cis double bonds in prolycopene, leading to all-trans-lycopene. Lycopene-β-cyclase (LCY-β) introduces two β-ionone rings in all-trans-lycopene, yielding all-trans-β-carotene. LCY-β and lycopene-ε-cyclase (LCY-ε) introduce a β- and an ε-ionone ring, respectively to produce α-carotene. Hydroxylases (HYD) convert the cyclic carotenes α- and β-carotene into all-trans-lutein and all-trans-zeaxanthin, respectively. All-trans-zeaxanthin is reversibly epoxidated into all-trans-violaxanthin. The epoxidation and de-epoxidation are catalyzed zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), respectively. Neoxanthin synthase produces the final product of the β-branch, all-trans-neoxanthin, from all-trans-violaxanthin. The inset shows the numbering of C atoms of β-carotene (Jia et al., 2017).
1.1.1. Carotenoids cleavage dioxygenases (CCDs) and apocarotenoids formation

CCDs are a family of non-heme iron-dependent enzymes present in all taxa. These enzymes catalyze the oxidative cleavage of double bonds in carotenoids to form apocarotenoids. Apocarotenoids are usually named according to the number of the C-atom at the cleavage site of the carotenoid precursor as a prefix, while the suffix indicates their functional group (e.g. β-apo-10’-carotenal for an aldehyde, a product of β-carotene cleavage). Some CCDs catalyze a secondary cleavage reaction by cleaving apocarotenoids, which are frequently further modified by various enzymes to yield bioactive compounds, such as hormones and signaling molecules (Ahrazem et al., 2016; Walter and Strack, 2011). The conjugated double bond system in a carotenoid backbone is very susceptible to oxidative cleavage that can take place without enzymatic activity, induced by the attack of reactive oxygen species which can be triggered by light (Ramel et al., 2012). However, targeted and specific cleavage of carotenoids is typically catalyzed by CCDs.
CCDs are structurally conserved; suggested by the elucidation of the crystal structures of three different CCDs from plants, bacteria, and mammals. These are Viviparous14 (Vp14) from Zea mays (Messing et al., 2010), the cyanobacterial, retinal-forming, apocarotenoid-15,15-oxygenase from Synechocystis spp. SynACO (Kloer et al., 2005); and retinal isomerohydrolase; retinal pigment epithelium protein of 65 kDa (RPE65) from Bos taurus (Kiser and Palczewski, 2010). Analysis of crystal structure data of these enzymes reveals that they share a common chain fold and have similar active centers (Harrison and Bugg, 2014; Sui et al., 2016). The tertiary structure of CCDs consists of a rigid seven-bladed β-propeller capped by α–helices, an active site with the catalytic iron coordinated by four histidine residues, and loops that form the substrate-binding

Figure 2. Crystal structure of three CCDs. *Synechocystis* ACO (left), maize VP14 (center) and bovine RPE65 (right). The ferrous catalytic iron is colored in orange. Secondary structural elements consisting of α-helices and β-sheets are colored in blue and green, respectively (Sui et al., 2013).
tunnel that leads from the active site to the protein exterior (Sui et al., 2016). The active center is located on the top side of the propeller near its axis. In addition, there are hydrophobic patches surrounding the active site tunnels (Sui et al., 2013). α-helix, β-sheets and loop regions bring the seven blades of the propeller together, by connecting β-strands within each blade (Figure. 2), (Sui et al., 2013).

The unique active site with catalytic iron coordinated by four conserved histidine residues plus three glutamine residues, which form a second coordination sphere, distinguishes CCDs from other iron-requiring enzymes. The ferrous iron cofactor is located near the top face of the propeller and is strictly required for cleavage activity. The role of Fe$^{2+}$ is to activate triplet oxygen needed for the oxidative cleavage of carotenoid and apocarotenoid substrates (Kowatz et al., 2013).

Carotenoids and apocarotenoids, the substrates for CCDs, are generally lipophilic. Thus, they normally accumulate in water-free environments within the cell. Therefore, the hydrophobic patches surrounding the active site tunnels are needed to mediate the substrate binding and regulate its availability (Golczak et al., 2010; Sui et al., 2013). The non-polar patches consist of hydrophobic amino acid residues that expand from the loop region to facilitate binding to membranes and extracting hydrophobic substrates (Auldridge et al., 2006). Patches are believed to dip into the membrane to enable the uptake and transfer of the substrate to reach the catalytic iron in the active site of the enzyme (Kloer et al., 2005; Kowatz et al., 2013).

The hydrophobic tunnels of all three described CCDs extend from the external surface of the protein to enter the active center passing by the iron and ending at the interior with a leucine residue. The tunnels are lined with non-polar residues and surrounded by non-polar patches to
serve as channels or outlets for the passage of lipophilic substrates (Kloer et al., 2005; Sui et al., 2013). Moreover, tunnels facilitate the hydrophobic interactions between the enzyme and the substrate preceding the substrate cleavage. Sequence alignment studies showed that the substrate binding tunnels of different CCDs differ considerably in their amino acid compositions. These differences result in geometric and steric variations in the binding region, which were found to be the cause of the stereo and regio-selectivity of CCDs. The hydrophobic residues in the substrate-binding region not only determine the specificity but also ensure the correct orientation of the substrate binding. Additionally, variations of amino acid compositions lead to different levels of stability of the enzyme-substrate complex at the steady state of the reaction (Sui et al., 2016).

Despite the numerous biochemical in vitro studies, which involved isotope labeling experiments, the mechanism of CCDs cleavage reaction remained controversial. However, there is a substantial body of evidence indicating that all carotenoid cleavage oxygenases act as dioxygenases (Harrison and Bugg, 2014).

In the monooxygenase reaction, only one of the two oxygen atoms of molecular oxygen is introduced into the substrate, forming an epoxide intermediate (Figure 3, left), while the other atom is reduced to water. In contrast, in the dioxygenase reaction, both O-atoms of atmospheric oxygen attack the double bond at the substrate cleavage site to form a dioxetane, an unstable intermediate, which rapidly breaks down into two aldehyde products (Figure 3, right) (Jia et al., 2017; Sui et al., 2013).
Figure 3. Monooxygenase and dioxygenase catalytic mechanisms proposed for carotenoid cleavage enzymes. In the monooxygenase reaction (left), an epoxide is formed with the involvement of one oxygen atom from atmospheric oxygen. Only one oxygen remains in the aldehyde products with the other derives from water. In the dioxygenase reaction (right), an unstable dioxetane intermediate is formed, and both dioxygen atoms remain in the aldehyde products (Sui et al., 2013).
Plant CCDs are classified into subfamilies according to their substrate preference, the cleavage site, and stereospecificity. Based on the Arabidopsis CCD family, plant CCDs are divided into five subfamilies CCD1, CCD4, CCD7, CCD8 and nine-cis-epoxy-carotenoid dioxygenases (NCEDs) (Walter and Strack, 2011). The first discovered carotenoid cleavage enzyme was the NCED Viviparous 14 (VP14) that was identified based on the corresponding maize ABA-deficient mutant. VP14 catalyzes the initial step in ABA biosynthesis; it specifically cleaves the 11, 12 double bond of 9-cis-epoxycarotenoids to produce the ABA precursor xanthoxin (Giuliano et al., 2003; Schwartz et al., 1997).

CCD1 enzymes cleave different cyclic and acyclic carotenoids and apocarotenoids at several double bond positions, leading to a variety of products with different chain lengths (Ilg et al., 2014). Unlike CCD1s, CCD4s cleave β-ionone ring containing cyclic carotenoids at the 9′, 10′ or the 7′, 8′ positions and produce C_{10} or C_{13} volatiles, such as cyclocitral or β-ionone, and the corresponding C_{30} or C_{27} apocarotenoids, respectively (Bruno and Al-Babili, 2016; Bruno et al., 2015; Rodrigo et al., 2013).

CCD7s and CCD8s act sequentially in SL biosynthesis, leading to carlactone (CL), the central intermediate in SL biosynthesis (Alder et al., 2012). CCD7 and CCD8 functions are conserved among several plant species. For instance, MAX4 from Arabidopsis, RMS1 from peas, DAD1 from petuniae and D10 from rice are true CCD8 orthologs (Al-Babili and Bouwmeester, 2015; Felsenstein, 1985; Foo et al., 2005; Waters et al., 2012). Likewise, MAX3, RMS5, DAD3, and D17 catalyze the same reaction (Booker et al., 2004; Vogel et al., 2010). The involvement of CCD7 and CCD8 in SL biosynthesis is also evolutionarily conserved. A recent study showed that a moss, Physcomitrella patens, also synthesizes carlactone by the action of CCD7 and then CCD8 (Decker et al., 2017).
There are many CCDs in different plant species with unidentified enzymatic activity. For example, the rice genome encodes thirteen CCDs, including several with unknown functions. Characterization of some of these rice enzymes is one of the aims of this thesis.

1.1.2. Biological functions of apocarotenoids

Carotenoids are vital not only in their intact form, but also as precursors of apocarotenoids, essential bioactive compounds found in bacteria, fungi, animals, and plants. Central cleavage of β-carotene (C\textsubscript{40}) yields retinal (C\textsubscript{20}), the vision chromophore in animals (Moise et al., 2014; Von Lintig, 2012). In plants, apocarotenoids serve as pigments, chromophores, aroma and scent constituents and, more important, as hormones and signaling compounds (Walter et al., 2010; Xie et al., 2010). A notable example of apocarotenoids pigment is bixin, a pigment of commercial value derived from the cleavage of lycopene at 5, 6 and 5’, 6’ double bonds. Bixin is obtained from the seeds of *Bixa orellana* and is used as a food colorant and for other industrial purposes. Crocetin is a further apocarotenoid pigment that is responsible for the color of the stigmas of *Crocus sativus*. Crocetin is derived from zeaxanthin cleavage at the C7, C8 and C7’, C8’ double bonds, which also leads to 3-OH-cyclocitral the precursor of safaranal and picocrocin that contribute to saffron taste (Moraga et al., 2009). C\textsubscript{13} cyclohexenone and C\textsubscript{14} mycorradicin, the cleavage products of C\textsubscript{40} carotenoid, are abundant apocarotenoids in mycorrhizal roots and are assumed to play a key role in regulating mycorrhization. The two plant hormones, abscisic acid (ABA) and strigolactones (SLs), are probably the most important and best-investigated apocarotenoids in plants (Walter et al., 2010). Furthermore, there are other apocarotenoids involved in a remarkable number of important biological processes and many more with yet unidentified functions (Domonkos et al., 2013).
1.2. **Strigolactones**

SLs are known as signaling molecules secreted by plant roots and released into the rhizosphere, initiating both parasitic and symbiotic interactions between plants and root parasitic weeds and between plants and symbiotic obligate heterotrophs, respectively (Ruyter-Spira et al., 2013; Xie et al., 2013). SLs owe their name to their ability to stimulate the germination of root parasitic plants of the genus *Striga* (Latin for “witch”) (Akiyama et al., 2005; Butler, 1994; Cook et al., 1966). However, SLs were later identified as a novel class of plant hormones that regulate many aspects of plant growth and development (Gomez-Roldan et al., 2008; Umehara et al., 2008). Since then, researchers have taken a great interest in explaining the hormonal function of SLs, exploring their diverse developmental and stress-related roles, identifying their receptors and elucidating their biosynthesis pathway.

1.2.1. **SLs are signaling molecules**

1.2.1.1. **SLs initially discovered as parasitic seed germination stimulators**

Although the phenomenon that parasitic seeds germinate only when they are close to the host plant root has been well-known for a long time, the chemicals that induce the seed germination were not identified until the sixties of the 20th century when strigol was discovered (Ha et al., 2014; Yoneyama et al., 2010). It was Cook’s team who first isolated and identified the stimulant produced in root exudates of cotton in 1966, which was achieved through a series of chromatographic techniques. They also confirmed that this compound is capable of triggering seed germination at a very low concentration. Butler (1995) named the strigol-related compounds Strigolactones.

Root parasitic plants have different host specificities. *Striga* species mainly infest cereal crops such as maize, rice, and sorghum, while *Orobanche* species infest sunflowers, tomatoes, carrots,
etc. *Striga* infestation causes a massive reduction in crop yield; affecting millions of people and raising a major food security concern, particularly in Sub-Saharan Africa (Parker, 2009). Root parasitic weeds are completely or partially incapable of performing photosynthesis, and their seeds have very limited reserves (Delavault et al., 2017). Hence, root parasitic weeds are obligate parasites that require a host for survival even at an early seedling stage. In addition, seeds of these parasites are characterized by a strong dormancy and the ability to survive in soil for decades (Delavault et al., 2017; Dörr, 1997). In other words, these seeds will survive dormant for a long time and germinate only when they perceive SLs in the soil, indicating the presence of a host plant (Andrew J. Humphrey, 2006; Dörr, 1997; Khosla and Nelson, 2016; Tsuchiya et al., 2010; Xie, 2016; Zwanenburg et al., 2016). After germination, the survival of the seedlings depends on the ability to find a host plant and connect to it within a few days (Andrew J. Humphrey, 2006; Tsuchiya et al., 2010). Root parasitic weeds are difficult to control because their life cycle is mostly underground and when they emerge above soil it is usually too late, as they become tightly bound to the host roots (López-Ráez et al., 2009) (Figure 4). Therefore, a proper understanding of the germination process and the nature of the stimulant is an important step towards controlling these pests.
1.2.1.2. **SL promotes arbuscular mycorrhizal fungi hyphal branching**

SLs are involved in another type of rhizospheric communication. SLs released by plants in the rhizosphere enhance the symbiosis between plant roots and arbuscular mycorrhizal fungi (AMF). In this symbiosis, the plant supplies the fungi with carbohydrates, and the fungi help the plant to get water and nutrients from the soil (primarily phosphate that has low soil mobility) and improve the host resistance to pathogens and drought (Akiyama et al., 2005; Andrew J. Humphrey, 2006; Bonfante and Genre, 2015; Dörr, 1997). This symbiosis is achieved by the incorporation of the fungal hypha into the host plant roots in a way that extends the plant root system to reach further into the soil (Parniske, 2008). This kind of beneficial symbiosis is very common in nature found in about 80% of land plants (Ruyter-Spira et al., 2013). AMF colonization is triggered in response to an insufficient phosphorous supply that induces the secretion of SLs, enabling the fungal partner to detect the host roots (Akiyama et al., 2005). The release of even a very low concentration of SLs in the soil is a sufficient plant recognition signal for the fungus. This signal triggers the hyphal branching of the AMF, creating contact to host roots to establish the symbiosis (Kohlen et al., 2011).

1.2.2. **Strigolactones are phytohormones**

Plants are sessile, thus, they need to regulate and adapt their growth in response to external stimuli. Phytohormones control the growth and development of plants as well as their response to biotic and abiotic stress (Bari and Jones, 2009; Peleg and Blumwald, 2011).

SLs were not considered as plant hormones until the discovery of their role in inhibiting shoot branching in 2008 (Gomez-Roldan et al., 2008; Umehara et al., 2008). Before that, other hormones, namely auxin, and cytokinines, were thought to be the main hormones that control shoot branching, as their pleiotropy had masked the function of SLs (de Saint Germain et al.,
Gomez et. al., 2008 studied high-branching plant mutants deficient in the shoot multiplication signal (SMS), a postulated long distance mobile signal controlling shoot branching by inhibiting lateral bud outgrowth. Surprisingly, direct application of the synthetic SL analog, growth regulator 24 (GR24) to SMS deficient mutants (ccd8 mutants) rescued the high-branching phenotype, while the SMS perception mutant max2 (more axillary growth2) did not respond to this treatment. Other SMS deficient mutants, ccd7 and max1, showed the same response as ccd8 mutants (Gomez-Roldan et al., 2008).

The role of SLs as shoot branching inhibitors was established through several physiological studies and grafting experiments on mutants with increased shoot branching (Mouchel and Leyser, 2007). These mutants were from various plant species, including; max in Arabidopsis (Booker et al., 2005), ramosus (rms) in peas (Beveridge et al., 1996; Sorefan et al., 2003) dwarf (d) and high tillering dwarf (htd) in rice (Arite et al., 2007; Zhou et al., 2013), and decreased apical dominance (dad) in petuniae (Drummond et al., 2012; Hamiaux et al., 2012).

In the meanwhile, it has been established that SLs play an important role in the control of different aspects of plant development, not just inhibiting shoot branching and that SLs regulate plant growth and architecture in accordance with nutrients, particularly phosphate, availability. For instance, SL-deficient and SL-insensitive Arabidopsis mutants have shorter primary roots and more lateral roots compared with WT plants, suggesting the role of SLs in determining root architecture.
SLs also accelerate leaf senescence, induce mesocotyl elongation, increase stem thickness and stimulate root hair elongation (Al-Babili and Bouwmeester, 2015; Brewer et al., 2013; de Saint Germain et al., 2013; Delaux et al., 2012; Flematti et al., 2016; Hoffmann et al., 2014; Ruyter-Spira et al., 2013; Seto et al., 2014; Smith, 2014; Smith and Li, 2014; Waldie et al., 2014; Waters et al., 2017) (Figure 5).
1.2.3. SLs structure and nomenclature

Natural SLs are carotenoid-derivatives composed of two moieties, a tricyclic lactone (ABC-rings) in canonical SLs, such as strigol and orobanchol, or less conserved structures in non-canonical ones, such as methyl carlactonoate, that is connected to the second conserved moiety, a butenolide (D-ring), by an enol ether bridge in R-configuration (Andrew J. Humphrey, 2006; Ruyter-Spira et al., 2013). There are approximately 25 characterized SLs. Canonical SLs differ through substitutions on the A- and B-rings such as methylation, hydroxylation or epoxidation (Al-Babili and Bouwmeester, 2015; Jia et al., 2017). In addition, they are divided based on the stereochemistry of the B/C-junction into the orobanchol- (C-ring in α-orientation) and strigol-like (C-ring in β-orientation) SLs (Figure 6) (Al-Babili and Bouwmeester, 2015). Strigol and orobanchol were named based on their parasitic weed specificity: where the former is more active towards seeds of *Striga* species and the latter shows better activity towards *Orobanche* species (Al-Babili and Bouwmeester, 2015).
1.2.4. SLs are synthesized from carotenoids

The similarity between the A-ring in SLs and the β-ionone ring in cyclic carotenoids led to the assumption that SLs might be carotenoid-derivatives. This assumption was then confirmed by studying several maize mutants and carotenoid biosynthesis inhibitors (Matusova et al., 2005).
A detailed study of maize carotenoid biosynthetic mutants and analysis of their root exudates, combined with the application of fluoridone, an inhibitor of the carotenoid biosynthesis pathway, revealed the carotenogenic origin of SLs (Matusova et al., 2005). The pathway of SL biosynthesis was elucidated after the discovery of the SL hormone function, which unraveled the involvement of the carotenoid cleavage dioxygenases 7 and 8, D27 and MAX1 in this process (Abe et al., 2014; Alder et al., 2012; Zhang et al., 2014) (Figure 7).
Figure 7. SLs biosynthesis. Initially, D27 isomerizes all-trans-β-carotene into 9-cis-β-carotene, which is cleaved by CCD7 into 9-cis-β-apo-10’-carotenal (C_{27}) and β-ionone (C_{13}). CCD8 converts 9-cis-β-apo-10’-carotenal into CL (C_{19}) and the C_{8}-compound ω-OH-(4-CH_{3}) heptanal. CL is then modified by CYP450 enzymes. MAX1, a CYP450 in Arabidopsis, catalyzes the successive oxygenation of CL to carlactonoic acid via 19-hydroxy-carlactone. Carlactonoic acid is further converted by an unknown methyl transferase into methyl carlactonoate, the substrate of LBO to form an unidentified hydroxylated methyl carlactonoate. Carlactone oxidase (CO), rice MAX1 homologue, Os900 catalyzes repeated oxygenation and ring closures to form 4-deoxyorobanchol (ent-2’-epi-5-deoxystrigol) from CL, then orobanchol synthase (OS) also a rice MAX1 homologue, Os1400, converts 4-deoxyorobanchol to orobanchol. In sorghum (left), 5-deoxystrigol is converted by an unknown CYP450 into sorgomol, the major SL in sorghum. Biosynthetic enzymes are illustrated in blue (Jia et al., 2017).
1.2.5. Strigolactone Biosynthesis Enzymes

1.2.5.1. D27

Lin et al. 2009, showed that D27, a protein with unknown function, is required for SL biosynthesis. This conclusion was drawn from the SL deficient phenotype of the rice d27 mutant, which could be rescued by exogenous application of the SL analog GR24. However, the study did not address the enzymatic activity of D27 nor its order in the pathway. Several grafting experiments were conducted on Arabidopsis D27 to determine its order in the pathway. It was found that D27 is likely acting upstream of MAX1 in the SL biosynthesis pathway (Waters et al., 2012). In addition, D27 proteins are equipped with a plastid transit peptide, suggesting their localization within this organelle. This, along with the strict stereo-specificity of CCD7 and the fact that D27 does not have homology to any known enzyme, led to the hypothesis that D27 works as an isomerase upstream of CCD7 and CCD8 to provide the substrate for CCD7 in the required stereo-configuration (Alder et al., 2012).

To test this hypothesis, Alder et al. studied the enzymatic activity of OsD27 in vivo and in vitro. The in vivo experiment was carried out by expressing OsD27 in E. coli cells engineered to accumulate β-carotene. They found a clear increase in the 9-cis/all-trans ratio, which indicates that OsD27 is a β-carotene 9-cis/trans isomerase. To confirm this result in vitro, they incubated OsD27 with each of these β-carotene isomers. Results obtained in this study demonstrated that D27 catalyzes reversible isomerization of all-trans-β-carotene into 9-cis-β-carotene, the substrate of CCD7.

A recent study of OsD27 activity shed light on the substrate preference of this enzyme. This study revealed that reversible isomerization catalyzed by OsD27 leads to an equilibrium of all-
trans-β-carotene: 9-cis-β-carotene in a 2:1 ratio. These data were obtained from HPLC analysis of in vitro incubation of recombinant D27 with each β-carotene isomer (Harrison et al., 2015).

Further investigations of D27 enzymatic activity, led by the question of whether other substrates of CCD7 (9-cis-zeaxanthin and 9-cis-lutein; see below) are also produced by D27, revealed that the enzyme has narrow substrate specificity. OsD27 substrates are C₄₀ bicyclic carotenoids wherein the presence of at least one un-substituted β-ionone ring is required (Bruno and Al-Babili, 2016). Therefore, β-carotene, α-carotene and β,β-cryptoxanthin were isomerized while zeaxanthin and violaxanthin were not. OsD27 neither accepted apocarotenoids nor acyclic or monocyclic substrates, such as lycopene or γ-carotene (Bruno and Al-Babili, 2016). Furthermore, OsD27 did not show isomerization activity with 15-cis- or 13-cis-β-carotene in vitro, suggesting strict specificity for the C9, C10 double bond (Bruno and Al-Babili, 2016).

1.2.5.2. CCD7

MAX3 (AtCCD7) was identified by Brook et al. (2004) as a key enzyme required for the synthesis of a novel carotenoid-derived shoot branching inhibitor. They reported that AtCCD7 cleaves the C9’, C10’ and/or C9, C10 double bonds in different carotenoids, including β-carotene, in vivo, to produce the C₁₃ volatiles, such as β-ionone. Schwartz et al. (2004) reported that AtCCD7 cleaves all-trans-β-carotene to generate the C₂₇ all-trans-β-apo-10’-carotenal, which is further cleaved by AtCCD8 to produce the C₁₈ compound, β-apo-13-carotenone that was believed to be the precursor of the signaling molecule that regulates lateral branching, later identified as SL. In addition, CCD7 tomato ortholog; SlCCD7 was reported to have similar activity (Vogel et al., 2010). Moreover, an in vitro study confirmed that AtCCD8 cleaves all-trans-β-apo-10’-carotenal into β-apo-13-carotenone and unidentified dialdehyde product (Alder et al., 2012).
However, exogenous application of β-apo-13-carotenone did not restore the wild-type phenotype of high-tillering rice ccd8 mutant (Alder et al., 2012), indicating that β-apo-13-carotenal is likely not the precursor of SLs. This result led to re-visiting the enzymatic activity of AtCCD7 and its homologues from rice and peas by Alder et al. (2012) who demonstrated that CCD7 enzymes are strictly stereospecific and cleave 9-cis-β-carotene, but no other β-carotene stereoisomer. This cleavage reaction resulted in the formation of β-apo-10′-carotenal and β-ionone.

The substrate specificity of CCD7 was further studied by (Bruno et al., 2014) who performed in vitro incubation of CCD7 from rice (Oryza sativa), garden peas (Pisum sativum) and Arabidopsis thaliana with different isomers of carotenoids other than β-carotene. This study showed that CCD7 enzymes have wide substrate-specificity and confirmed their strict-stereo and regio-specificity. AtCCD7 and PsCCD7 also cleave other 9-cis-configured carotenoids, such as 9-cis-lutein and 9-cis-zeaxanthin, resulting in the formation of 9-cis-3-OH-ε-apo-10′-carotenal and 9-cis-3-OH-β-apo-10′-carotenal, respectively (Bruno et al., 2014). Another recent in vitro study investigated the AtCCD7 activity on acyclic carotenoids. AtCCD7 converts 9-cis-ζ-carotene, 9′-cis-neurosporene, and 9-cis-lycopene, into the corresponding 9-cis- apocarotenals; 9-cis-ζ-apo-10′-carotenal and 9-cis-apo-10′-lycopenal, respectively (Bruno et al., 2016). The fact that CCD7 can cleave 9-cis-zeaxanthin, -lutein, and the corresponding acyclic carotenoids raised the question of whether CCD8 can convert other apocarotenals produced by CCD7 into CL-like compounds that may be precursors of yet unidentified, hydroxylated SLs or SLs with ε-ionone ring.

CCD7 enzymes are also involved in the biosynthesis of other biologically important apocarotenoids. For instance, some evidences indicate that CCD7 action is required for the production of apocarotenoids that accumulate to high levels in roots upon mycorrhizal
colonization. These apocarotenoids are glycosylated $C_{13}$ cyclohexenone and linear $C_{14}$ mycorradicin derivatives. Both classes derive from $C_{40}$ carotenoid via oxidative cleavage at the C9, C10, and C9’, C10’ positions. This origin was demonstrated by the application of a carotenoid biosynthesis inhibitor (norflurazon), and by studying mutants affected in carotenoid biosynthesis. Additionally, carotenoid biosynthesis is induced in mycorrhizal roots, while carotenoids occur only in traces in non-mycorrhizal roots (Fester et al., 2002). Vogel et al. (2010); used tomato (Solanum lycopersicum) plants that form symbiotic relationships with AMF and compared them to tomato plants expressing a $SlCCD7$ antisense construct. The objective was to study the role of $SlCCD7$ in the production of arbuscular mycorrhiza-induced apocarotenoids. Their results confirmed the involvement of $SlCCD7$ in SLs as well as in $C_{13}$ and $C_{14}$ apocarotenoids biosynthesis. They found that arbuscular mycorrhiza-induced accumulation of $C_{13}$ and $C_{14}$ apocarotenoids is inhibited in $SlCCD7$ antisense lines. Both cyclohexenone and mycorradicin derivatives were greatly reduced in colonized antisense lines roots, compared to wild-type roots, supporting the indications that CCD7 is required for their synthesis. Another study showed that mycorrhizal $rms5$ (CCD7) mutant plants of peas have reduced levels of cyclohexenone and mycorradicin derivatives, compared to mycorrhizal wild-type plants (Floss et al., 2008).

1.2.5.3. CCD8

CCD8 is the third enzyme in the SL biosynthesis pathway. CCD8 enzymes, from different plant species, can cleave all-trans-$\beta$-apo-10’-carotenal in vitro to produce $\beta$-apo-13-carotenone (Schwartz et al., 2004). Understanding the stereospecificity of CCD7 substantiated that all-trans-$\beta$-apo-10’-carotenal is not a product of CCD7 as it was thought to be (Alder et al., 2012; Bruno et al., 2014). When CCD8 was incubated with 9-cis-$\beta$-apo-10’-carotenal, the true product
of CCD7, it produced a new and interestingly different C\textsubscript{19} molecule; an enol ether lactone which resembles SLs in its structure and number of carbons. This compound was given the name carlactone. By putting the pieces together, they generated a scheme showing the reactions leading from β-carotene to CL (Figure 7).

CCD8 has multiple catalytic activities. To produce β-apo-13-carotenone, CCD8 catalyzes a typical carotenoid cleavage reaction that occurs via unstable intermediate dioxetane, as generally described for CCDs (Sui et al., 2013), while converting 9-\textit{cis}-β-apo-10′-carotenal to CL takes place through isomerization, rearrangements, and unusual double oxygenation in one step. Catalyzing two oxidative cleavages in one reaction is a unique feature of this enzyme (Alder et al., 2012; Harrison et al., 2015). Alder et al. also confirmed that the CL skeleton is entirely derived from 9-\textit{cis}-β-apo-10′-carotenal and their conversion rate data showed that the 9-\textit{cis} isomer is the preferred substrate for CCD8 (Alder et al., 2012).

Whether CCD8 is mono- or dioxygenases was unknown. The catalytic mechanism and kinetics of CCD8 were studied by developing a pre-steady-state kinetic analysis using UV-visible stopped-flow experiments (Harrison et al., 2015). The results showed that CCD8 has two kinetic steps in the catalytic cycle, which likely corresponds to the two consecutive oxygenation steps as well as it could correspond to physical steps such as substrate binding. A recent study conducted by Bruno et al. (2017), proposed a mechanism for the CCD8-catalyzed CL formation and identified the instable second product that should arise simultaneously with carlactone (C\textsubscript{19}) from 9-\textit{cis}-β-apo-10′-carotenal (C\textsubscript{27}), which had not been identified in the study of Alder et al. (2012). They incubated 9-\textit{cis}-β-carotene with \textit{Pisum sativum} enzymes PsCCD7 and PsCCD8 in the presence of \textsuperscript{18}O\textsubscript{2} / H\textsubscript{2}\textsuperscript{16}O. The obtained data showed that CCD8 needs only two substrates to produce CL; 9-\textit{cis}-β-apo-10′-carotenal and molecular oxygen (O\textsubscript{2}). Moreover, they demonstrated
that the second product is a small, unstable C₈ molecule (x-OH-(4-CH₃) heptanal) that has an aldehyde and an alcohol group. Identification of this unstable compound was achieved using a proper derivatization agent that enabled its detection. The $^{18}\text{O}_2$ and $\text{H}_2^{16}\text{O}$ labeling experiments demonstrated that all three oxygen atoms in CL molecule originate from atmospheric O₂, which require the consumption of two O₂ molecules. These findings imply that CCD8 dioxygenase reaction occurs twice with the 9-cis-β-apo-10’-carotenal, unlike the single cleavage that takes place with the all-trans substrate (Bruno et al., 2017). Furthermore, CCD8 is highly stereospecific, as it produces CL exclusively in the $R$ configuration, explaining why all natural SLs have this stereo-configuration at the corresponding C2’-atom (Flematti et al., 2016; Seto et al., 2014).

### 1.2.6. Carlactone, the precursor of SLs

CL is the central intermediate in SL biosynthesis and is a biologically active compound that stimulates *Striga* seeds germination and inhibits shoot branching, resembling the functions of SLs (Alder et al., 2012). Several studies demonstrated that CL is the precursor of canonical and non-canonical SLs (Seto et al., 2014; Zhang et al., 2014). In fact, CL structure appears to be only a few steps away from 5-deoxystrigol. These steps include dioxygenation, dehydrogenation and ring closure (Figure 7), closing the gap between canonical SLs and β-carotene (Alder et al., 2012). Seto et al. (2014) showed that Arabidopsis *max1* mutants accumulate 700 fold more CL, compared to the wild-type, indicating that MAX1 may act directly on CL to convert it into the next metabolite in the pathway.

CL was later identified as an endogenous compound. Seto et al. (2014) confirmed the presence of CL *in planta* by detecting it in Arabidopsis. In addition, CL was also identified as an *in vitro* product of the combined activity of CCD7 and CCD8 in moss; a recent study investigated
the formation of CL in the moss *Physcomitrella patens*, by studying the activity of *PpCCD7* and *PpCCD8* *in vitro*. They demonstrated that CCD7 and CCD8 from *Physcomitrella patens*, can produce CL, indicating that the biosynthesis of CL is evolutionarily conserved (Decker et al., 2017).

1.2.7. From CL to various SLs

The following sections deal with the conversion of CL to canonical and non-canonical SLs.

1.2.7.1. MAX1

MAX enzymes belong to the Cytochrome P450 superfamily. CYP450 are heme-containing mono-oxygenases requiring molecular oxygen and NADPH for their action (Benveniste et al., 1991) and are present in animals, plants, fungi, and bacteria. CYP450s catalyze a wide range of reactions, including hydroxylation, oxygenation, epoxide formation, and mediated ester cleavage and dehydrogenation (Halkier, 1996). In eukaryotic cells, these enzymes are mainly present in the endoplasmic reticulum (Benveniste et al., 1991).

To investigate the role of MAX1 in CL conversion, a feeding experiment of labeled CL was performed. $^{13}$C-labeled CL was fed to Arabidopsis *max4/ccd8* mutants, resulting in the formation of a $^{13}$C-labeled non-canonical SL identified as methyl carlactonoate (Abe et al., 2014; Seto et al., 2014). Similarly, feeding labeled CL to the rice *d10/ccd8* mutant resulted in the formation of labeled 4-deoxyorobanchol (2′-epi-5-deoxystrigol) and orobanchol. These findings suggest that MAX1s are required for CL conversion (Abe et al., 2014; Seto et al., 2014).

Zhang et al. (2014) co-expressed rice MAX1s with CL biosynthesis enzymes (OsD27, OsCCD7, and OsCCD8) in *Nicotiana benthamiana* leaves by *Agrobacterium tumefaciens* mediated transformation. Upon analyzing by LC-MS/MS, they found a significant decrease of CL content
concurred by the production of ent-2-epi-5-deoxystrigol (4-deoxyorobanchol), indicating that the OsMAX1 homologue they tested, Os900, catalyzes the oxidation of CL and that it is enantiomer specific. For further confirmation, they expressed OsMAX1 homologues in a yeast system and incubated the microsomal preparations with CL produced in vitro. Microsomes isolated from yeast expressing Os900 showed ent-2-epi-5-deoxystrigol (4-deoxyorobanchol, Figure 7) formation when analyzed by LC-MS/MS. These findings are in agreement with the results obtained by transient expression in Nicotiana benthamiana. Another MAX1 homologue, Os1400, was characterized as orobanchol synthase, which catalyzes the hydroxylation of 4-deoxyorobanchol to produce orobanchol (Figure 7) (Zhang et al., 2014). Carlactonoic acid (CLA) is a product of Arabidopsis MAX1 formed by oxidation of CL via the intermediate, 19-hydroxy CL, in vitro (Abe et al., 2014). In rice, carlactonoic acid is converted into 4-deoxyorobanchol and orobanchol. These findings were demonstrated by feeding experiments (Abe et al., 2014).

1.2.7.2. Other downstream enzymes

A recent study identified Lateral Branching Oxidoreductase (LBO) as a gene with an expression pattern similar to MAX3 and which encodes an oxoglutarate-dependent oxidoreductase that catalyzes a later step in SL biosynthesis. The lbo mutant showed a more-branching phenotype, and grafting experiments with SL mutants indicated that LBO acts downstream of MAX1 that produces CLA. Accordingly, lbo contained elevated levels of CLA and methyl carlactonoate (MeCLA). In addition, LBO consumes MeCLA but does not act on CL and CLA (Brewer et al., 2016). However, the product formed by LBO is still unknown, though LC-MS analysis suggests it is a hydroxylated MeCLA (Brewer et al., 2016).
Gobena et al. (2017) provided evidence that loss of function of the gene LOW GERMINATION STIMULANT 1 (LGS1), codes for an enzyme recognized as sulfotransferase, is responsible for changing of the dominant SL in sorghum root exudates from 5-deoxystrigol to orobanchol. 5-deoxystrigol is highly active Striga germination stimulant which has opposite stereochemistry of orobanchol (Gobena et al., 2017).

1.3. Objectives

1.3.1. Biosynthesis and Identification of Hydroxycar lactone

The main focus of the first part of this thesis is to investigate if plants produce new CLs that derive from β-carotene or other carotenoids and, if so, whether these intermediates have a biological function(s). This question was raised by the wide substrate specificity of CCD7 enzymes that cleave different 9-cis-configured carotenoids, including 9-cis-zeaxanthin, yielding 9-cis-configured apocarotenoids, such as 9-cis-3-OH-β-apo-10’-carotenal. To answer this question, we used an in vitro approach to test CCD8 activity with different 9-cis-configured apocarotenoids, such as 9-cis-3-OH-β-apo-10’-carotenal.

1.3.2. Enzymatic study of rice dioxygenase ZAS

The second objective deals with a rice CCD with unknown function, which was later acknowledged as ZAS. The aim was to study the enzymatic activity of ZAS. Therefore, we investigated the function of this enzyme and identified its product, using recombinant protein for in vitro incubations. A further objective was to define ZAS preferred substrate by conducting a conversion rate study. Also, we aimed to determine if the ZAS product is a natural metabolite by verifying its presence in planta.
2. Material and Methods

2.1 Material

2.1.1. Chemicals

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<td>Petrolether (PE)</td>
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<tr>
<td>SYBR safe DNA gel stain</td>
<td>Invitrogen</td>
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<tr>
<td>Tert-butylmethyl-ether (TBME), HPLC-grade</td>
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<tr>
<td>Trihydroxymethylaminomethan (Tris)</td>
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<tr>
<td>Tris(2-Carboxyethyl)phosphine Hydrochloride</td>
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<td>Triton X-100</td>
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<td>Tween 20</td>
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<td>Yeast extract</td>
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2.1.2. Carotenoids and apocarotenoids

<table>
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<th>Company</th>
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<tbody>
<tr>
<td><em>all-trans</em>-β-carotene</td>
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<tr>
<td>Apo-8’-lycopenal</td>
<td>BSAF</td>
</tr>
<tr>
<td>Apo-12’-lycopenal</td>
<td>BSAF</td>
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<tr>
<td>β-apo-12’-carotenal</td>
<td>BSAF</td>
</tr>
<tr>
<td>β-apo-10’-carotenal</td>
<td>BSAF</td>
</tr>
<tr>
<td>β-apo-8’-carotenal</td>
<td>BSAF</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>BSAF</td>
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<td>Lycopene</td>
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<tr>
<td>Retinal</td>
<td>BSAF</td>
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<tr>
<td>Zeaxanthin</td>
<td>BSAF</td>
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<td>--------------------------------</td>
<td>------------</td>
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<tr>
<td>3-OH-β-apo-8′-carotenal</td>
<td>BSAF</td>
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<tr>
<td>3-OH-β-apo-10′-carotenal</td>
<td>Buchem</td>
</tr>
<tr>
<td>3-OH-β-apo-13′-carotenal</td>
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<tr>
<td>3-OH-9-cis-β-apo-10′-carotenal</td>
<td>Buchem</td>
</tr>
<tr>
<td>3-OH-9-cis-α-apo-10′-carotenal</td>
<td>Buchem</td>
</tr>
<tr>
<td>9-cis-β-carotene</td>
<td>Carotenature</td>
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<tr>
<td>9-cis-β-apo-10′-carotenal ^13C</td>
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<tr>
<td>9-cis-α-apo-10′-carotenal</td>
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### 2.1.3. Cloning materials

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<tr>
<td>DNase I, Amplification Grade</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>dNTP</td>
<td>New England Biolabs</td>
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<tr>
<td>DTT (Dithiothreitol)</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Gateway® LR Clonase® II Enzyme mix</td>
<td>GoldBio</td>
</tr>
<tr>
<td>Gel Extraction Kit</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>iScript™ cDNA Synthesis</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>LR Clonase II</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Minipreps, DNA purification system</td>
<td>Qiagen</td>
</tr>
<tr>
<td>One Kb Plus DNA Ladder</td>
<td>Invitrogen</td>
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<tr>
<td>One Shot® TOP10 Chemically Competent E. coli</td>
<td>Invitrogen</td>
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<tr>
<td>Power SYBR® Green PCR Master Mix</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>Phusion® High-Fidelity DNA Polymerase</td>
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<td>Plasmid Midiprep Kit</td>
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<tr>
<td>Restriction enzymes</td>
<td>New England Biolabs</td>
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<tr>
<td>RNeasy Kit</td>
<td>Qiagen</td>
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<tr>
<td>RNeasy Plant Mini Kit</td>
<td>Qiagen</td>
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<tr>
<td>T4-DNA-ligase</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Taq-DNA-Polymerase</td>
<td>Life Technologies</td>
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2.1.4. Primers

<table>
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<th>Primer</th>
<th>Restriction site</th>
<th>Sequence (5′–3′)</th>
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<td>AtD27-Agro-Forward</td>
<td>NcoI</td>
<td>catgCCATGGCAATGAACACTAAGCTATCCTTCTCA</td>
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<td>AtD27-Agro-Reverse</td>
<td>NsiI</td>
<td>atttGCGGCCGCCTATACTCTCTTACTCCGAGCT</td>
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<td>AtMAX3-Agro-Forward</td>
<td>NcoI</td>
<td>atttGCGGCCGCcatGTCTCTCCCTATCCCGCC</td>
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<tr>
<td>AtMAX4-Agro-Reverse</td>
<td>ScaI</td>
<td>CgtagtcTaATTTGTGAAAGTGAAAGCAATA</td>
</tr>
<tr>
<td>AtMAX4-Agro-Forward</td>
<td>XhoI</td>
<td>CCGctcgagATGGCTTTTTGATCAACCCA</td>
</tr>
<tr>
<td>AtMAX4-Agro-Reverse</td>
<td>NotI</td>
<td>atttgcggcgcTTAATCTTTGGGGATCCAGCAA</td>
</tr>
<tr>
<td>OsHYD 100- Forward</td>
<td>BamHI</td>
<td>attGGATCCAtggccgtcgcgaggctg</td>
</tr>
<tr>
<td>OsHYD 100- Reverse</td>
<td>NotI</td>
<td>atttGCGGCCGCATCACCGCGCTTAATCTCTTATAATGAGGTA</td>
</tr>
<tr>
<td>OsHYD 500- Forward</td>
<td>BamHI</td>
<td>ctagGATCCAtggccgtgcgcgg</td>
</tr>
<tr>
<td>OsHYD 500- Reverse</td>
<td>NotI</td>
<td>atttGCGGCCGCATCACCGCGCTTAATCTCTTATAATGAGGTA</td>
</tr>
<tr>
<td>ZAS-MBP-Forward</td>
<td>Recombinational-</td>
<td>CCAGGGAGCAGCCTCGATGACAGCTCTCTCTCTCTGATCAGGT</td>
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<tr>
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<td>Recombinational-</td>
<td>GCAAGGCACCAGGCTCTGATCAGC TTGTGATGAGGTA</td>
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2.1.5. Media

2.1.5.1. Media for bacteria

**LB-media (1L):**

Bacto-Peptone 10 g  
Yeast extract 5 g  
NaCl 10 g  
pH 7.5

**2 YT-media (1L):**

Bacto-Trypton 16 g  
Yeast extract 10 g  
NaCl 5 g  
pH 7.5
### 2.1.5.2. Media for plants

**Modified Hoagland nutrient solution**

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<tr>
<th>Salt</th>
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<th>g/mol</th>
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<tr>
<td>NH$_4$NO$_3$</td>
<td>5.6</td>
<td>80.04</td>
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<tr>
<td>K$_2$HPO$_4$.3H$_2$O</td>
<td>0.4</td>
<td>228.2</td>
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<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.8</td>
<td>246.48</td>
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<tr>
<td>K$_2$SO$_4$</td>
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<td>174.2</td>
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<tr>
<td>FeSO$_4$.7H$_2$O</td>
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<tr>
<td>Na$_2$EDTA.2H$_2$O</td>
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<td>CaCl$_2$.2H$_2$O</td>
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<tr>
<td>KNO$_3$</td>
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**Micronutrients**

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<tr>
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<td>MnCl$_2$.4H$_2$O</td>
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<td>CuSO$_4$.5H$_2$O</td>
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<td>Na$_2$MoO$_4$.2H$_2$O</td>
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2.1. Methods

2.1.1. 3-H-CL identification and biosynthesis

2.1.1.1. In vitro assays

The OsCCD8 cDNA was expressed as thioredoxin-fusion in *E.coli* BL21 cells harboring HSP60/HSP70 plasmid which encodes the groES–groEL chaperone system and controlled by an arabinose-inducible promoter. A single colony of the transformed *E.coli* was cultured overnight. From the overnight culture (0.5 mL) was inoculated into 50 mL media and grown at 28°C to OD 0.5 at 600 nm then induced by 0.2% (w/v) arabinose and grown again shaking at 28°C for four hours. Cells were harvested by centrifugation and resuspended in lysis buffer (sodium phosphate buffer pH 8 containing 1% Triton X-100 and 10 mM of dithiothreitol). Lysozyme was added to the suspension and incubated on ice for 30 min. After that, the crude lysate was sonicated and centrifuged at 12000 rpm and 4°C for 10 min. The supernatant containing the protein was collected to be used for the *in vitro* incubation with the substrate. Synthetic substrates were purchased from Buchem B. V. (Apeldoorn, Netherlands). Substrates (9-cis-β-apo-10-carotenal, 3-OH-9-cis-β-apo-10-carotenal, 3-OH-β-apo-10’-carotenal, β-apo-10’carotenal, 9-cis-α-apo-10 carotenal, and 3-OH-9-cis-α-apo-10-carotenal) were quantified spectrophotometrically, at their individual λ<sub>max</sub> using extinction coefficient (Barua and Olson, 2000). Dried substrate was resuspended ethanolic detergent mixture 0.4% (v/v) Triton X-100. The mixture was then dried using a vacuum centrifuge and resuspended in the incubation buffer (2 mM tris 2-carboxyethyl phosphine, 0.4 mM FeSO4 and 2 mg/ml catalase (Sigma, Deisenhofen, Germany) in 200 mM Hepes/ NaOH, pH 8). OsCCD8 crude cell lysate, 50 μl of the soluble fraction of overexpressing cells, was added to the assay. The assay was incubated overnight shaking at 140 rpm at 28°C in the dark. Next day, the reaction was stopped by adding two volumes of acetone and the lipophilic
compounds were separated by partition extraction with petroleum ether: diethyl ether 1: 4 (v/v),
dried, and resuspended in methanol for HPLC analysis.

2.1.1.2. HPLC analysis

For 3-H-CL identification, preparation and purification, we used the following separation
systems. For 3-H-CL identification, we used separation system I; reversed phase C\textsubscript{18} column
(ZORBAX Eclipse XDB-18. 4.6×150 mm. 3.5 μm) in Ultimate 3000 UHPLC system developed
at a flow rate of 1 mL/min with a gradient from 25% acetonitrile and 75% water to 100%
acetonitrile within 20 min. For preparative isolation of 3-H-CL, we performed two purification
steps. In the first step, we used the separation system II; reversed phase C\textsubscript{18} column (ZORBAX
Eclipse XDB-18. 4.6×150 mm. 5 μm) developed at a flow rate of 1 mL/min, from 50%
acetonitrile and 50% water to 100% acetonitrile in 19 min. For the second purification step, we
employed separation system III, YMC Carotenoid C\textsubscript{30} column (4.0×250 mm, 5 μm) operated at a
flow rate of 0.7 mL/min and a temperature of 30°C. The run started with isocratic elution with
5% (methanol:tert-butyl methyl ether (1: 1, v/v)) and 95% (methanol:water:tert-butyl methyl
ether (30: 10: 1, v/v/v)) for 10 min then it reached 100% (methanol:tert-butyl methyl ether (1: 1,
v/v)) in 5 min and maintained these conditions for 4 min.

2.1.1.3. Nuclear magnetic resonance (NMR) spectroscopy analysis.

For NMR analysis, approximately 1 mg was dissolved in 40 μL of CD\textsubscript{2}Cl\textsubscript{2}. The NMR
experiments were performed on a Bruker AVANCE II spectrometer operating at a \textsuperscript{1}H resonance
frequency of 500.13 MHz. The instrument is equipped with a 1.7 mm TXI triple (\textsuperscript{1}H, \textsuperscript{13}C, \textsuperscript{31}P)
microprobe with a z-gradient coil and with pulse angles of 5.9 μs (\textsuperscript{1}H) and 14.9 μs (\textsuperscript{13}C),
respectively. Bruker TOPSPIN software (version 3.2, patch level 3) was used to acquire and
process the NMR data. To verify the structure of 3-H-CL, 1D $^1$H and 2D homo-and heteronuclear NMR techniques have been applied.

All experimental work and data analysis were kindly done by Dr. Martina Vermathen, Department of Chemistry & Biochemistry, and University of Berne, Switzerland (See supplementary data).

2.1.2. Detection of 3-H-CL in planta

2.1.2.1. Transient expression of SLs biosynthesis enzymes cDNA in Nicotiana benthamiana

Plasmids for transient expression in *Nicotiana benthamiana* (pBIN-OsD27, pBIN-OsCCD7, and pBIN-OsCCD8), harboring the indicated cDNAs under the control of the CaMV 35S promoter, were kindly provided by Prof. Harro Bouwmeester, Lab. of Plant Physiology, University of Amsterdam.

To generate the plasmids for transient expression in *Nicotiana benthamiana*, full-length cDNA of *AtD27* (AT1G03055), *AtMAX3* (AT2G44990) and *AtMAX4* (AT4G32810) were amplified by Phusion polymerase (New England Biolabs) from Arabidopsis Col-0 leaf cDNA using primers (See primers table section 2.1.4.). The PCR products were first cloned into pJET1.2 (Fermentas). After sequence confirmation, the fragments were digested with desired restriction sites and ligated into the linearized entry vector pIV1A_2.1 which contains a CaMV35S promoter (www.pri.wur.nl/UK/products/ImpactVector/). After sequence confirmation of the pIV1A_2.1 entry clones, Gateway LR clonase II enzyme mix (Invitrogen) reactions were performed to transfer the fragments into the pBinPlus binary vector (van Engelen et al., 1995), generating p35S:PBIN-AtD27, p35S:PBIN-AtMAX3 and p35S:PBIN-AtMAX4.
The resulting plasmids of rice (OsCCD7, OsCCD8, and OsD27) and Arabidopsis (AtD27, AtMAX3, and AtMAX4) were introduced into *Agrobacterium tumefaciens* strain GV3101 competent cells. Positive Agrobacterium colonies were selected for further infiltration. Liquid Agrobacterium cultures of different genes were grown at 28 °C at 200 r.p.m. for 2 days in LB medium with antibiotics corresponding to the binary vectors. Cells were harvested by centrifugation for 15 min at 4,000 r.p.m. at room temperature and then resuspended in 10 mM MES-KOH buffer (pH 5.7) and 10 mM MgCl₂ containing 100 mM acetosyringone then the tubes were kept rolling in the dark at room temperature for 2-4 hours. Concentrations of the agrobacterium strains harboring different constructs were fixed at a final OD600 of 0.5. In all experiments, an agrobacterium strain harboring a gene encoding the TBSV P19 protein was added to enhance protein production by suppression of gene silencing. *Nicotiana benthamiana* plants were grown in soil pots in the greenhouse with 16 h light at 25 °C and 8 h dark at 28 °C. Construct combinations in agrobacterium were infiltrated into leaves of 5-week-old *Nicotiana benthamiana* plants by using a 1 mL syringe. Leaves of the same stage were selected to minimize the variability. For each gene combination, three leaves from each plant were infiltrated, and three plants were used for individual biological replicates. The bacterial suspension in buffer was slowly injected into the abaxial side of the leaf to spread to the whole leaf area (Zhang et al., 2014). Five days after agro-infiltration, leaves were harvested, lyophilized and prepared for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis.

**2.1.2.2. Detection of 3-H-CL in Arabidopsis thaliana mutants**

To investigate whether 3-H-CL is a natural metabolite, we tried to detect the presence of endogenous 3-H-CL in Arabidopsis. *Arabidopsis thaliana* Col-0 background wild-type in
addition to the following mutants; _max1_ mutant which is defective in cytochrome P450, _max4_ mutant which is defective in CCD8, _max2_ mutant which is defective in SL response, and _dl4_ mutant which is SL-insensitive mutant were tested. Arabidopsis seedlings were grown hydroponically in half strength modified Hoaglands nutrients solution (pH 5.8) for 3 weeks under normal conditions then phosphate starvation was applied for one week. After that, shoots and roots were collected separately to be extracted and analyzed by LC-MS/MS.

2.1.2.3. Detection of 3-H-CL in rice seedlings

To detect the presence of endogenous 3-H-CL in rice, we tested the following cultivars; Shiokari wild-type and the high SLs producer, IAC rice, in addition to _dl4_ mutant. Rice seedlings were grown hydroponically in half strength modified Hoaglands nutrients solution (pH 5.8) for 2 weeks in the growth chamber (PGR15, Controlled Environment Limited, Canada) for two weeks under normal conditions. Then we grew them for one week under phosphate starvation to induce SLs production. After that, shoots and roots were collected separately for extraction and LC-MS/MS analysis.

2.1.2.4. 3-H-CL extraction from plant tissue

For CL and SL analysis in _Nicotiana benthamiana_ and _Arabidopsis thaliana_, 50 mg of fine-ground tissue was used. 3-H-CL was extracted by ethyl acetate containing D$_3$-3-OH-β-apo-13-carotenone as an internal standard amber glass vial. The vials were vortexed and sonicated for 15 minutes in a Branson 5510 ultrasonic bath (Branson Ultrasonics). Samples were centrifuged at 3800 rpm for 8 minutes, after which the solvent was transferred to an 8 mL glass vial. The pellets were re-extracted with ethyl acetate. Then the combined ethyl acetate supernatants were dried using LabconcoRapidVap.
2.1.2.5. 3-H-CL purification using silica SPE

To purify 3-H-CL, the crude extracts were dissolved in a mixture of ethyl acetate and n-hexane and loaded onto a pre-equilibrated column (HyperSep silica 500mg/3mL SPE, precondition with 3 mL ethyl acetate, and then 3 mL n-hexane). Subsequently, the columns were washed with 3 mL of n-hexane and 3-H-CL was eluted with 3 mL of ethyl acetate. Samples were evaporated to dryness, and the residues were dissolved in acetonitrile: water (90:10, v/v). The samples were filtered through 0.22 μm PVDF filters before LC-MS/MS analysis.

2.1.2.6. 3-H-CL LC-MS/MS analysis

Analysis of 3-H-CL in plant material was performed on a Dionex Ultimate 3000 UHPLC system coupled with a Q-Exactive plus MS (Orbitrap detector, Thermo Scientific) with an electrospray source. Chromatographic separation was carried out on a Phenomenex Gemini C18 (150×2.0 mm, 5 μM) column, at 35 °C. The mobile phase (A and B) was (0.1% formic acid- 95% acetonitrile - 5% water) and (0.1% formic acid- 95% water- 5% acetonitrile), respectively. The gradient used was 20 min, 25%-100% A; 20-25 min, 100% A; 25-26 min, 100%-25% A; 26-36 min, 25% A. The flow rate was 0.2 mL/min. The MS was operated in positive ionization mode. The conditions were as follows: spray voltage 4.0 KV, auxiliary gas heater temperature 310 °C, sheath gas flow rate 30 μL/min, auxiliary gas flow rate 10 μL/min, and PRM (capillary temperature 320 °C, S-lens RF level 55, resolution 70,000, HCE 15 eV).

2.1.3. Investigation of 3-H-CL precursor in planta

2.1.3.1. Transient expression of β-carotene hydroxylases cDNA in *Nicotiana benthamina*

Rice β-carotene hydroxylases (BCHs), *OsHYD100* (NCBI accession XM_015758320) and *OsHYD500* (NCBI accession XM_015773287.1), cDNAs were cloned in the binary vector; pBINPlus (van Engelen et al., 1995) for transient expression in *Nicotiana benthamiana*. cDNAs
were amplified by PCR using primer pairs tagged with Histidine (HIS tag). The sequence of hemagglutinin (HA tag) was included in the reverse primers (See primers table section 2.1.4.)

Plasmids pThio-DanII-OsHYD100 and pThio-DANII-OsHYD500, which include the indicated cDNAs (obtained as synthetic genes from Genescript and constructed by Mark Bruno, University of Fribourg), were used as templates for PCR. The amplified fragments were inserted into pCRTM8 TOPO® TA vector to create entry clones. The PCR8-OsHYD100-HIS and PCR8-OsHYD500-HIS were cloned in to the binary vector pBIN using Invitrogen Gateway LR clonase enzyme to yield pBIN-OsHYD100-HIS and pBIN-OsHYD500-HIS, respectively. The transient expression of β-carotene hydroxylases cDNA in *Nicotiana benthamina* was performed as detailed in section 2.1.2.1. Extraction and detection of 3-H-CL was carried out as explained in sections, 2.1.2.4-2.1.2.6.

### 2.1.3.2. Quantification of zeaxanthin in *Nicotiana benthamina* leaves infiltrated with BCH

#### 2.1.3.2.1. Carotenoid extraction *Nicotiana benthamina* leaves

Lyophilized leaves were grind to fine powder using (MINIBEADBEATER, Biospec). About 15 mg was weighed and extracted with 2 ml acetone. Samples were vortexed and sonicated for 15 min in a Branson 5510 ultrasonic bath (Branson Ultrasonics) then centrifuged at 3800 rpm for 8 minutes, after which the solvent was transferred to an 8 mL glass vial. The pellets were reextracted with acetone until pellet is nearly colorless. Then the combined acetone supernatants were dried using LabconcoRapidVap and resuspended in of MeOH. Saponification was performed for separation of carotenoids from chlorophyll by adding KOH solution (60 %, w/v) and heating under reflux for five min at 70 °C. PE/DE (2:1, v/v) was added. The organic phase containing carotenoids was again separated from the aqueous chlorophyll phase and dried in the
rotatory evaporator. Carotenoids were re-suspended in CH$_2$Cl$_2$ and filtered through 0.22 μm PVDF filters before HPLC analysis.

2.1.3.2.2. HPLC analysis for zeaxanthin quantification

For carotenoid analysis from plant tissues, separation was performed using a reversed phase YMC C$_{30}$ column (150 × 3 mm, 5 μm, YMC Europa, Schermbeck, Deutschland) developed at flow rate of 0.6 mL/min and maintained at 30°C with a gradient from 25 % to 100 % A (A, MeOH/TBME, 1:1, v/v; and B, MeOH/H$_2$O/TBME, 5:2:1, v/v/v) within 13 minutes and maintaining the final conditions for 1 minute.

2.1.4. 3-H-CL bioassays

2.1.4.1. Parasitic seed germination bioassays *Striga hermonthica*

*Striga* seeds were first separated from sand residue and organic debris using the sucrose gradient (40% and 60%). Cleaned seeds were then surface-sterilized using (50%) sodium hypochlorite with 0.4% of Tween-20 for 10 min. Subsequently, seeds were thoroughly rinsed using sterile MilliQ water through a glass vacuum filter holders and flask (Fisher Scientific) under a laminar flow cabinet. The sterilized seeds were then allowed to dry under the same cabinet. Approximately 50 to 100 seeds were evenly spread on sterilized glass fiber filter paper discs. The discs were placed in 90 mm Petri-dishes (12 discs per Petri-dish) on Whatman filter paper moistened with 3 mL sterile MilliQ water. The Petri dishes were sealed, wrapped in aluminum foil and placed in an incubator at 30°C for 12 days. The discs with preconditioned seeds were allowed to dry under laminar flow cabinet. The discs were then placed in another Petri-dish (six per Petri-dish) containing a filter paper ring, wetted with sterile MilliQ water. 50 μL of each of 3-H-CL solutions (0.0001, 0.001, 0.01, 0.1, 1 and 10 μM) were added per disc, replicated four times for each concentration. In addition, GR24 solutions containing the same concentrations
were used as a positive control (rac-GR24 was purchased from Chiralix, the Netherlands). Sterile MilliQ water was used as a negative control in each germination assay. After application, *Striga* seeds were incubated again at 30°C in the dark for two days. Germination (seeds with radicle emerging through the seed coat) was assessed using a binocular microscope. The germination rate (GR) in percentage was calculated for each disk (replication) using the formula:

$$\text{GR} (\%) = \left( \frac{\text{Ngs}}{\text{Nts}} \right) \times 100$$

Where Ngs is the number of germinated seeds per disc, Nts is the total number of seeds per disk.

### 2.1.4.2. Tillering bioassay

The rice seeds *cv Shiokari* (WT) and *d10 (ccd8 mutant)* (Ishikawa et al., 2005) were surface sterilized with 50% bleach for 12 minutes, then the bleach was removed by five subsequent washings with sterilized water. The seeds were incubated in sterile water at 28°C in the dark for 2 days to germinate. The germinated seeds were transferred to Petri-dishes with filter paper moistened with 10 mL 0.5 MS sterilized medium. Then the plates were kept in a growth cabinet at 28°C under fluorescence white light (250 μM m⁻² s⁻¹) for 5 days. The seedlings were then transferred to the hydroponic system. Each seedling was grown in 15 mL tube containing half-strength modified Hoagland’s nutrients solution (+P, pH 5.8). After two days, seedlings were fed via their roots with 3.5μM 3-H-CL on daily basis for two weeks. At the end of the second week, rice tillers were counted and the length of the second tiller was measured for each seedling.
2.2.5. Rice Dioxygenase (ZAS); enzymatic study

2.2.5.1. In vitro assays

We investigated the enzymatic activity of three rice CCD8 homologues, accession numbers; XP015611401.1, XP015648368.1, and XP015649272.1; which we identified as ZAS, ZASL1, and ZASL2, respectively.

Full-length ZAS cDNA was produced from total rice RNA by RT-PCR. The cDNA lacking the stop codon was then amplified and ligated into pBAD/Thio-TOPO (Invitrogen), enabling the expression of the enzyme fused with thioredoxin at the N-terminus and equipped with a C-terminal 6-His-tag. The integrity of the obtained plasmid pThio-ZAS his was confirmed by sequencing. The fusion protein was expressed in E. coli BL21 cells. Bacterial growth and induction and the crude cell lysate preparation was carried out as explained in the CCD8 in vitro assay section 2.1.1.1.

To study the enzymatic activity of ZAS and ZAS-likes, crude cell lysate was incubated in vitro with the following carotenoids and apocarotenoids; β-carotene, 9-cis-β-carotene, zeaxanthin, lycopene, 3-OH-β-apo-10’-carotenal, 3-OH-β-apo-8’-carotenal, 3-OH-β-apo-12’-carotenal, β-apo-10’-carotenal, β-apo-8’-carotenal, 9-cis-α-apo-10-carotenal, 3-OH-9-cis-α-apo-10-carotenal, 9-cis-β-apo-10-carotenal, 3-OH-9-cis-β-apo-10-carotenal, apo-10-lycopena, and apo-8-lycopenal (Figure 8). Synthetic substrates were purchased from BASF, Germany and Buchem B. V. Apeldoorn, Netherlands (See table 2.1.2.). Substrates were quantified, prepared, and incubated with the crude lysate in the assay buffer according to the in vitro assay protocol detailed in section 2.1.1.1.
Figure 8. Structures and names of carotenoids and apocarotenoids tested *in vitro* as potential substrates for ZAS.
2.2.5.2. HPLC and LC/MS analysis

To analyze ZAS in vitro assays sample, YMC Carotenoid (250×4.0 mm, 5 μm) C30 column was used in Ultimate 3000 U-HPLC system developed at a flow rate, 0.6mL/min and a column temperature of 30°C. Solvents used were A (methanol:tert-butylmethyl ether (1: 1, V/V)) and B (MeOH:water:tert-butylmethyl ether (30: 10: 1, V/V/V)). The run starts with 100% B that goes to 45% in 15 minutes and stays at 45% for 9 minutes.

For LC/MS analysis, UHPLC-Q-Orbitrap was used for identification of zaxinone by comparing retention time, MS/MS fragments and their accurate mass with the standard.

2.2.5.3. ZAS conversion rate study

Conversion rate study was done using in vitro assays, stopped by freezing in liquid nitrogen at nine different time points during 2 hours of incubation. A standard curve for the product was constructed from which the product concentrations were calculated. The areas of the peaks in the HPLC analysis of the assays were corrected according to the internal standard to account for the loss due to extraction and HPLC analysis. Analysis of ZAS in vitro assays sample was performed on Ultimate 3000 UHPLC system with a YMC Carotenoid C30 column (250 × 4.0 mm, 5 μm) at a flow rate of 0.6 ml/min and a column temperature of 30°C. Mobile phases included methanol:tert-butylmethyl ether (1:1, v:v, A) and methanol:water:tert-butylmethyl ether (30:10:1, v:v:v, B). The gradient started from 100% B to 45% B in 15 min followed by isocratic elution with 45% B for 9 min. Retinal was used as an internal standard in the HPLC quantitative analysis. An exponential model using Origin Software was performed to fit the data.

2.2.5.4. Identification and quantification of ZAS second product; C9 dialdehyde

To confirm that ZAS cleaves the C27, 3-OH-β-apo-10’-carotenal specifically at 13, 14 double bond to form the C18 product, zaxinone, we identified the second product, a C9 dialdehyde.
2.2.5.4.1. Derivatization; conjugating to PFBHA

C9-dialdehyde was identified by using LC-MS via a derivation reaction with aldehyde-selective derivatization reagent O-(2,3,4,5,6 Pentafluorobenzyl) hydroxylamine hydrochloride. This compound is used for aldehyde and ketone derivatization as the carbon from the carbonyl group is electropositive and considering that the methyl ketones and aldehydes do not present steric hindrance, the aminenucleophile located at the end of the PFBHA molecule could easily attack the carbonyl group, forming an oxime as a final product (Figure 9). The same reaction scheme can be applied to the synthetic C10 dialdehyde (used as internal standard) and the final product is an analogue oxime. Three technical replicas were prepared and the overall ratio between the peak area of the derivatized C9 dialdehyde and C10 derivatized standard was taken in the control and ZAS samples. For this, 60 μM of apo-8,15’carotindial (C10 dialdehyde) was dissolved in 200 μL of CHCl3:MeOH (2:1) and added to each sample, vortexted, and then sonicated 3 times for 3 seconds. The liquid layer was extracted and transferred to a new tube. The samples were dried in a vacuum centrifuge. Once they were completely dry, they were dissolved in 100μL of methanol and 50 μL of PFBHA (25mg/1mL MeOH) was added. The samples were incubated at 35°C for 1 hour, allowing the reaction to take place. After 1 hour, the samples were dried again; the content was dissolved in 50μL of DMSO and then analyzed by LC/MS.
Figure 9. The derivatization of C\textsubscript{9} dialdehyde with PFBHA. C\textsubscript{9} dialdehyde (Apo-14, 10-carotindial) was conjugated to O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride after incubating ZAS with C\textsubscript{27}, 3-OH-\beta-apo-10'-carotenal for one hour.
2.2.5.4.2. Identification; LC/MS analysis

Analysis of C\textsubscript{9} dialdehyde was performed on a Dionex Ultimate UHPLC system coupled with a Q-Exactive plus MS. Chromatographic separation was carried out on a Phenomenex Gemini C18 column (150 × 2.0 mm, 5 μm) with the mobile phase of acetonitrile:water:formic acid (95:5:0.1, v:v:v, A) and acetonitrile:water:formic acid (5:95:0.1, v:v:v, B) at the flow rate of 0.2 mL/min and the column temperature of 35°C. The gradient was as follows: 0-20 min, 50% to 100% B, followed by washing with 100% B and equilibration with 50% B. The MS parameters were as follow: positive mode, spray voltage of 4.0 kV, auxiliary gas temperature of 310°C, sheath gas flow rate of 30 μl/min, auxiliary gas flow rate of 10 μl/min, capillary temperature of 320°C, S-lens RF level of 55, resolution of 70,000, and HCE of 15 eV.

2.2.5.4.3. Quantification of ZAS substrate and products

The formation of the product was monitored by calculating the ratio between the peak areas of derivatized C\textsubscript{9} in the control and ZAS samples. For proper and accurate quantification, an internal standard is needed to correct the values and account for the losses during the samples handling. For that, a customized synthetic C\textsubscript{10} dialdehyde was used as the internal standard.

For further confirmation, the evolution of the 3-OH-β-13-apocarotenal (C\textsubscript{18}) and the decrease in the 3-OH-β-apo-10'-carotenal C\textsubscript{27} were also monitored. For this, ZAS \textit{in vitro} assays (three replicates) were carried out and the samples were extracted as described in section 2.1.1.1.

Retinal (C\textsubscript{20}) was added as internal standard. The samples were analyzed by LC/MS and the ratio between the peak areas of the C\textsubscript{27} substrate and C\textsubscript{18} product compared to the peak area of retinal (C\textsubscript{20}) standard was taken in both control and ZAS samples.
2.2.5.5. *In vitro* assay with ZAS purified protein

To generate maltose-binding protein (MBP)-fusion protein, full-length ZAS cDNA was amplified by PCR from pThio-ZAS-His using the primers ZAS-MBP-F and ZAS-MBP-R (See primers table 2.1.4.) and cloned into the pET-His6 MBP N10 TEV LIC cloning vector (2C-T vector; http://www.addgene.org/29706/) with 6xHis and MBP tags at the N-terminus32. After sequence confirmation (KAUST, Bioscience Core Lab), the obtained plasmid, pET-MBP-ZAS, was then transformed into *E. coli* BL21 (DE3) cells. The cells were grown in LB broth containing ampicillin (100 mg/ml) at 37 °C until an OD600 of 0.5, and expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16 °C for 16 h shaking. Harvested cells were resuspended in lysis buffer: 50 mM Tris–HCl (pH 8.0), 200 mM NaCl, detergent (0.5% Triton X-100) and 2 mM DTT. After sonication (40% amplitude (10 min), 2 sec ON, 1 sec OFF) on ice for 10 min, the lysate was centrifuged at 25,000 rpm for 30 min at 4 °C. Further, the supernatant was allowed to bind to MBP-beads-Amylose Resin (New England BioLabs) for 2 hours at 4 °C. Then, washed 3 times with buffer (50 mM Tris–HCl (pH 8.0), 200 mM NaCl, 2 mM DTT) for 15 min each at 4 °C and eluted with 50 mM maltose monohydrate after shaking for 30 min at 4 °C. Purified fusion protein MBP-ZAS was concentrated using an Amicon 10K filter unit (Merck Millipore). Purification was monitored by SDS-PAGE analysis.

*In vitro* assays with crude lysates of MBP-ZAS fusion expressing cells were performed following the method described for crude assays with the thioredoxin-ZAS-His fusion. *In vitro* assays with purified protein were conducted at a protein concentration of 250 ng/µl (MBP-ZAS). Purified protein was added in a volume of 50 µl to the assay buffer containing substrate and detergent, and prepared as described above. Assays were extracted and analyzed as described in section 2.1.1.1.
2.2.5.6. Detection of zaxinone in planta

For the identification and quantification of zaxinone in planta, plant material was lyophilized and ground. 25 mg tissue powder spiked with 1.65 ng of D₃-zaxinone, was extracted with 2 ml of ethyl acetate twice with sonicating for 15 min in an ultrasonic bath and centrifuging for 8 minutes at 3800 rpm at 4°C. The two supernatants were combined and dried under vacuum. The residue dissolved in 100 µl of ethyl acetate and 2 ml of hexane for the following purification. Samples were run through a silica gel SPE column (500mg/3ml) preconditioned with 3 ml of ethyl acetate and 3 ml of hexane. After washing with 3 ml Hexane, zaxinone were eluted in 3 ml ethyl acetate and evaporated to dryness under vacuum. The residue was re-dissolved in 200 µl of acetonitrile: water (25:75, v:v) and filtered through a 0.22 µm filter for LC-MS/MS analysis. Qualitative analysis of zaxinone extracted from plant material was performed on a Dionex Ultimate 3000 UHPLC system coupled with a Q-Exactive plus MS (Thermo Scientific) with a heated-electrospray ionization source. Chromatographic separation was carried out on Phenomenex Gemini C₁₈ column (150×2.0 mm, 5 µm) with the mobile phase of water:acetonitrile:formic acid (95:5:0.1, v:v:v, A) and acetonitrile:formic acid (100:0.1, v:v, B) in a gradient program (0-20 min, 25%-100% B, followed by washing with 100% B and equilibration with 25% B). The flow rate was 0.2 ml/min. The injection volume was 10 µl, and the column temperature was maintained at 35°C for each run. The MS conditions were as follows: positive mode, spray voltage of 4.0 kV, auxiliary gas heater temperature of 310°C, sheath gas flow rate of 30 µl/min, auxiliary gas flow rate of 10 µl/min, capillary temperature of 320°C, S-lens RF level of 55, resolution of 70,000, and HCE of 15 eV for MS/MS. The quantification of zaxinone and apo-10'-zeaxanthinal in rice tissues was performed using HPLC-Q-Trap-MS/MS with MRM mode. Chromatographic separation was achieved on an Acquity
UPLC BEH C\textsubscript{18} column (50 × 2.1 mm; 1.7 μm; Waters) with mobile phases consisted of water: acetonitrile (95:5, v:v, A) and acetonitrile (B), both containing 0.1% formic acid. A linear gradient was optimized as follows (flow rate, 0.2 ml/min): 0–20 min, 25% to 100% B, followed by washing with 100% B and equilibration with 25% B. The injection volume was 5 μl, and the column temperature was maintained at 30°C for each run. The MS parameters were as follows: positive ion mode, ion source of turbo spray, ionspray voltage of 5500 V, curtain gas of 40 psi, collision gas of medium, gas 1 of 60 psi, gas 2 of 50 psi, turbo gas temperature of 400°C, declustering potential of 60 V, entrance potential of 10 V, collision energy of 20 eV, collision cell exit potential of 10 V.
3. Results

3.1. 3-OH-Carlactone (3-H-CL) identification and biosynthesis

3.1.1. CCD8 converts 3-OH-9-cis-β-apo-10’-carotenal to 3-H-CL in vitro

First, we tested whether rice CCD8 (OsCCD8) cleaves 9-cis apocarotenoid products of CCD7 other than 9-cis-β-apo-10’-carotenal, the precursor of CL. For this purpose, we tested hydroxylated, 9-cis-configured β-apo-10’-carotenal and α-apo-10’-carotenals as substrates in vitro. HPLC analysis of incubations with 9-cis-3-OH-β-apo-10’-carotenal, which can be formed by CCD7 from zeaxanthin, showed a new product accompanied by a significant reduction in the amount of substrate, compared to the control. Based on the activity of CCD8 in forming carlactone from 9-cis-β-apo-10’-carotenal, we assumed that the product detected in the incubation with 9-cis-3-OH-β-apo-10’-carotenal is 3-OH-carlactone (3-H-CL; Figure 10). The identity of this product was confirmed by LC-MS/MS based on its high-resolution MS and MS/MS data (Figure 11). We also investigated the activity of CCD8s from Pisum sativum (pea) and Arabidopsis thaliana. We found that 3-H-CL can also be produced by CCD8 from pea and Arabidopsis (Figure 10), indicating that this enzymatic activity is conserved among plants. In contrast, CCD8 of rice, pea and Arabidopsis did not show any activity when incubated with 9-cis-3-OH-α-apo-10’-carotenal and 9-cis-α-apo-10’-carotenal (Figure 12), indicating that CCD8 enzymes do not convert substrates that have an ε-ionone instead of β-ionone ring.
Figure 10. The biosynthesis of 3-H-CL. (A) HPLC analysis of CCD8s in vitro assays; Incubation of CCD8s from rice (OsCCD8), Arabidopsis (AtCCD8) and pea (PsCCD8) with 3-OH-9-cis-β-apo-10'-carotenal (I) formed a novel product, which we later identified as 3-H-CL (II). Chemical structures and UV/Vis spectra of substrates are depicted in the inset. (B) The proposed path from all-trans-β-carotene to 3-H-CL. It is likely that 9-cis-3-OH-β-apo-10'-carotenal is produced by CCD7 from 9-cis-zeaxanthin that can arise directly from 9-cis-β-carotene by hydroxylation of the β-ionone rings, that is catalyzed by β-carotene hydroxylases (HYD). It is not clear yet if the all-trans to 9-cis isomerization of β-carotene takes place before the hydroxylation or it is the other way around. Next, CCD8 converts 9-cis-3-OH-β-apo-10'-carotenal to 3-H-CL, which could be a precursor of new SLs.
Figure 11. Identification and confirmation of \textit{in vitro} produced 3-H-CL. (A) LC-MS analysis of 3-H-CL (Retention time: 13.57) produced \textit{in vitro} from 9-cis-3-OH-\textit{β}-apo-10'-carotenal by the action of OsCCD8. (B) Expected [M+H]⁺ molecular ion of C₁₉H₂₇O₄ (m/z 319.19009) from MS/MS spectrum indicated the elemental composition.
Detection and quantification of 3-H-CL in planta

After the confirmation of 3-H-CL biosynthesis in vitro, we investigated its biosynthesis in planta. We used Nicotiana benthamiana as an expression host to transiently express the SL biosynthetic genes D27, CCD7 and CCD8. First, we overexpressed rice SL biosynthetic genes in Nicotiana benthamiana leaves using Agrobacterium tumefaciens system and analyzed the level of 3-H-CL using LC-MS/MS. After 5 d of transient expression, 3-H-CL was detected when OsD27 was co-infiltrated with OsCCD7 and OsCCD8, suggesting that 3-H-CL can be synthesized in plants (Figure 13). Next, we co-infiltrated Arabidopsis AtD27 with AtCCD7 and AtCCD8 in tobacco leaves and expectedly 3-H-CL was detected in the samples (Figure 14). However, we did not detect 3-H-CL in tobacco leaves when SL biosynthetic gene was either overexpressed alone or in a combination of two (Figure 15). This shows that all three SL
biosynthesis enzymes are required for 3-H-CL production. Moreover, we detected a higher amount of CL compared to 3-H-CL (about 7 folds) which may indicate that CCD8 prefers the non-hydroxylated 9-cis-substrate (Figure 15).

To verify 3-H-CL precursor in planta, we coexpressed rice β-carotene hydroxylases 100 (OsHYD100) and 500 (OsHYD500), which hydroxylases β-carotene to produce zeaxanthin (Davison et al., 2002; DellaPenna and Pogson, 2006), with three rice SL biosynthesis enzymes. Upon co-expression of BCHs with OsD27, OsCCD7 and OsCCD8, the level of 3-H-CL was not increased, compared with samples infiltrated with OsD27, OsCCD7, and OsCCD8 simultaneously (Figure 15).

Furthermore, we analyzed the content of zeaxanthin in the samples infiltrated individually with OsHYD100 and OsHYD500, expecting an increase in zeaxanthin content in the infiltrated leaves, compared to the control. However, overexpression of BCHs did not lead to a significant increase in the zeaxanthin/β-carotene ratio (Figure 16).

These results did not support the hypothesis we proposed in figure (10B) about the involvement of BCHs in the biosynthesis of 3-H-CL precursor.
To further confirm the presence of 3-H-CL in rice, we used the roots of the SL perception mutant d14 as plant material which accumulates high levels of endogenous CL (Seto et al., 2014). For this purpose, we extracted 3-H-CL from the lypholized root tissue of rice d14 and analyzed it by LC-MS/MS. The results of LC-MS/MS analysis validated that roots of three weeks old rice d14 seedlings contain 3-H-CL. The MS spectra of endogenous 3-H-CL extracted from d14 roots matched the spectra of the in vitro produced 3-H-CL. This indicates that 3-H-CL is an endogenous metabolite in rice (Figure 17).
Figure 14. Identification of 3-H-CL in *Nicotiana benthamiana* leaves upon overexpression of Arabidopsis cDNAs coding for AtD27, AtCCD7, and AtCCD8, using UHPLC-MS/MS. (A) Extracted ion chromatogram of 3-H-CL extracted from tobacco leaves expressing the Arabidopsis enzymes (upper), and $^{13}$C-3-H-CL standard produced from *in vitro* assay (lower). (B) MS/MS spectrum of 3-H-CL formed in tobacco leaves by the Arabidopsis enzymes. The MS spectrum is inserted.
Figure 15. 3-H-CL and CL quantification in tobacco (*Nicotiana benthamiana*) leaves infiltrated with cDNA of rice SL biosynthesis genes; *OsD27*, *OsCCD7*, and *OsCCD8* individually and with combinations in addition to BCHs HYD100 and HYD 500. Data are mean ± SE of three replicates.
Figure 16. The relative abundance of zeaxanthin to β-carotene in tobacco leaves transiently over-expressing the rice β-carotene hydroxylases, OsHYD100 or OsHYD500. Leaves infiltrated with the empty vector (EV) were used as a control. Data were represented as mean (zeaxanthin/β-carotene) ± SEM (two-tailed Student t-test, P<0.05) of six independent replicates.
3.1.3. Structure verification by nuclear magnetic resonance analysis

To verify the structure of 3-H-CL (I) produced in vitro, 1D $^1$H, and 2D homo-and hetero-nuclear NMR techniques have been applied. The $^1$H- and $^{13}$C-chemical shift values found for (I) are summarized in Table 1, along with the experimental chemical shift values measured for the synthesized 3-H-CL. In addition, the values obtained from chemical shift prediction (Abrams et al., 1990) and experimental values reported for CL (Mori et al., 2016) are given in Table 1. The experimental chemical shift values matched well with the predicted ones (Supplementary Figures 1-8).

3-H-CL was chemically synthesized to be used as a reference. Professor Kohki Akiyama, Osaka Prefecture University, was able to synthesize 3-H-CL in nine steps (Supplementary Figure 9). A good agreement between the isolated in vitro product and the synthesized compound was found.

Figure 17. Identification of endogenous 3-H-CL from rice by using UHPLC-MS/MS. MS spectra of endogenous 3-H-CL extracted from $d14$ mutant root tissue (A) and $^{13}$C-3-H-CL standard produced from in vitro assay (B); PRM spectra of endogenous 3-H-CL with ion pairs at $m/z$ 319.19039>97.02879 (I) and $m/z$ 319.19039>147.11659 (III) extracted from $d14$ mutant root tissue; (C) $^{13}$C-3-H-CL with characterized ions pairs at $m/z$ 320.19374>98.03211 (II) and at $m/z$ 320.19374>147.11659 (III). The proposed structures of fragments are inserted.
Taken together, the NMR analysis demonstrates that the product formed by OsCCD8 from 9-\textit{cis}-3-OH-\textbeta-apo-10\textquotesingle-carotenal is 3-H-CL (Supplementary Figures 10-23).
Table 1: 1H and 13C NMR data of 3-hydroxy-carlactone

Experimental 1H and 13C chemical shifts for isolated and synthesized 3-H-CL dissolved in and referenced to CD$_2$Cl$_2$ (1H: 5.32 ppm, 13C: 54.0 ppm). For comparison, predicted 1H and 13C chemical shifts* for 3-hydroxy-carlactone and corresponding values reported for CL (Alder et al., 2012) are shown.

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[*] ACD Shift Prediction Software Release 11.00, Version 11.02 (2008)
3.2. 3-H-CL bioassays

3.2.1. 3-H-CL induced seed germination of *Striga hermonthica*

To investigate the biological activity of 3-H-CL as a germination stimulant, we applied different concentrations of 3-H-CL produced *in vitro* on preconditioned *Striga hermonthica* seeds and used GR24 as a positive control. After two days of treatment, the germinated seeds were counted. The application of 3-H-CL significantly increased *Striga* seeds germination, compared to those of the mock treatment (water) (Figure 18). This shows that 3-H-CL possesses SL-like activity and induces *Striga* seeds germination in a concentration-dependent manner.

![Figure 18](image-url)

Figure 18. Germination stimulating activity of 3-H-CL in *Striga hermonthica* seeds treated with different 3-H-CL concentrations in comparison to GR24 (1 µM). Data are means ± SE (n = 4); (P < 0.05, Tukey’s honest significant difference [HSD] test).
3.2.2. 3-H-CL restored the wild-type tillering phenotype of d10 mutant

The strigolactone deficient rice mutant d10/ccd8 has a high-tillering phenotype (Arite et al., 2007; Umehara et al., 2008). To investigate if 3-H-CL has an SL-like effect on rice tillers, we treated rice d10 mutant with in vitro produced 3-H-CL for two weeks. We counted the number of the tillers and measured the length of the second tiller in three-week-old rice seedlings grown in the hydroponic. The application of 3.5 μM 3-H-CL rescued the high tillering d10 mutant and this effect was similar to that of GR24 at 2.5 μM (Figure 19A). Two weeks treatment with 3-H-CL affected length of second tiller of d10 significantly and reduced this length just like wild-type. The length of second tillers was 10 times less in 3-H-CL treated d10 mutant as compared to mock. In addition, we recorded 90 mm length of the second tiller in d10-mock and application of 3-H-CL inhibited this length to 9 mm in d10 mutant which is comparable to GR24 and wild-type (Figure 19B).

![Figure 19](image_url)

Figure 19. Effect of 3-H-CL on d10 tillering. (A) Effect of H-CL on the growth of the second tiller (red arrows) in rice d10 mutant. Seedlings were treated with 3.5 μM H-CL or 2.5 μM GR24 for two weeks. (B) Length of second tiller of rice d10 mutant in response to 3-H-CL application. Rice seedlings were treated with 3.5 μM 3-H-CL for two weeks and then the second tiller length was measured. Bars represent means ± SE of nine replicates (right).
3.4. Enzymatic study of a new rice CCD

CCDs cleave carotenoids to form hormones and signaling molecules. In the present study, we investigated the enzymatic activity of a rice CCD encoded by *LOC_Os09g15240*. This gene belongs to undescribed plant CCD sub-family found in most plants. We studied the cleavage reaction of this enzyme and identified its product as a novel signaling molecule, zaxinone. Subsequently, we named the enzyme zaxinone synthase ZAS.

3.4.1. The phylogenic analysis of ZAS sub-family

To explore the distribution of ZAS in plants, we extracted 748 sequences representing all CCD genes in 67 genomes of land plant species including moss, fern, monocots, and dicots. Phylogenetic analysis suggests that ZAS represents a sixth CCD sub-family, besides the five known ones (Figure 20). *O. sativa* ZAS belongs to an accordingly designated cluster which also includes other three ZAS paralogues ZAS-L1, ZAS-L2, and ZAS-L3. Our *in vitro* study showed no cleavage activity for ZAS-L1 and ZAS-L2. The third homologue, ZAS-L3 is currently under investigation.
Figure 20. Neighbour-joining tree of 782 amino acid sequences of plant CCD orthologues. Bootstrap values only on nodes of NCED, CCD1, CCD4, ZAS, CCD7, and CCD8 clusters are shown. Circles of red, green, yellow and blue represent sequences of ZAS, ZAS-L1, ZAS-L2, and ZAS-L3, respectively. The scale bar indicates an estimated 0.1 change per amino acid (Wang et. al., under revision in Nature communication, 2018).
3.4.2. *In vitro* enzymatic study

To study the enzymatic activity of ZAS, ZAS-L1, and ZAS-L2, we expressed their respective cDNAs as thioredoxin-fusion in BL21 *E. coli* cells with the chaperone system, which is supposed to enhance the correct folding of the protein. The crude cell lysate was incubated *in vitro* with the following carotenoids and apocarotenoids; β-carotene, 9-*cis*-β-carotene, zeaxanthin, lycopene, 3-OH-β-apo-10’-carotenal, 3-OH-β-apo-8’-carotenal, 3-OH-β-apo-12’-carotenal, β-apo-10’-carotenal, β-apo-8’-carotenal, 9-*cis*-α-apo-10-carotenal, 3-OH-9-*cis*-α-apo-10-carotenal, 9-*cis*-β-apo-10-carotenal, 3-OH-9-*cis*-β-apo-10-carotenal, apo-10-lycopena, and apo-8-lycopenal (Figure 8). HPLC analysis revealed only an activity of ZAS with hydroxylated apocarotenoids. No cleavage product was detected with ZAS-L1 or ZAS-L2 assays (data not shown).

![Figure 21](image)

Figure 21. Enzymatic cleavage by ZAS. (A) UHPLC analysis showed that ZAS can cleave 3-OH-β-apo-10’-carotenal (apo-10’-zeaxanthinal) to produce 3-OH-β-apo-13-carotenal (zaxinone). (B) The enzymatic cleavage of C_{27} apo-10’-zeaxanthinal at 13, 14 double bond by ZAS.
In vitro experiments with carotenoids and apocarotenoids indicated that ZAS has higher activity towards 3-OH-β-apo-10'-carotenal (Figure 21). The product, 3-OH-β-13-apocarotenal (13-apo-zeaxanthinone) which we call zaxinone for the sake of simplicity, was found when ZAS was incubated with the following substrates; 3-OH-β-apo-10'-carotenal (we call it apo-10’-zeaxanthinal), 3-OH-β-apo-8'-carotenal, and 3-OH-β-apo-12'-carotenal (Figure 22).

Figure 22. Other hydroxylated substrates. ZAS cleaves 3-OH-apo-12'-carotenal (S1) and 3-OH-apo-8'-carotenal (S2) and the product detected was zaxinone (P).
For LC-MS/MS confirmation, UHPLC-Q-Orbitrap was used for identification of zaxinone by comparing retention time, MS/MS fragments and the accurate mass with the standard obtained from special synthesis (Figure 23).

![Figure 23](image)

3.4.3. ZAS substrate preference study

In order to determine the substrate preferences of ZAS, the conversion rates of the two estimated best substrates, apo-10’-zeaxanthinal and 3-OH-apo-8’-carotenal (apo-8’-zeaxanthinal), were measured in incubations with equal amounts of total protein crude lysate. Results showed that apo-10’-zeaxanthinal has three folds higher conversion rate than apo-8’-zeaxanthinal. After one
hour, ZAS converted almost all apo-10’-zeaxanthinal substrate to zaxinone, whereas only one-third of zaxinone was formed from apo-8’-zeaxanthinal, indicating apo-10’-zeaxanthinal is ZAS preferred substrate (Figure 24). However, we could not detect the expected second product, the C9-dialdehyde in the assays, likely due to its instability and the extraction/separation system used.

Figure 24. The conversion rate curve of apo-10’-zeaxanthinal (square) and apo-8’-zeaxanthinal (triangle) to form zaxinone catalyzed by ZAS. Data are mean of three replicates.
3.4.4. Identification and quantification of ZAS second product; C₉ dialdehyde

HPLC analysis did not enable the detection of the expected second cleavage product, the C₉-dialdehyde. Thus, no proof for a single double cleavage at the C13, C14 double bond was provided. To confirm that ZAS cleaves the C₂₇ apocarotenoid 3-OH-β-apo-10’-carotenal (apo-10’-zeaxanthinal) only at the 13, 14 double bond to form the C₁₈ product, zaxinone, we also identified the second product, a C₉ dialdehyde. For this purpose, we used O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride to conjugate (build a shiff base) carbonyl products present in incubations as demonstrated in (Figure 9). Upon derivatization, we detected the expected conjugated product C₂₃H₁₈O₂N₂F₁₀ (m/z = 541.09674) as well as the most abundant fragment; C₉H₁₁N₂ (m/z = 147.09155) by LC-MS (Figure 25). This result confirms that ZAS performs a single cleavage and that this cleavage is regio-specific at the C13, C14 double bond.
Figure 25. Identification and detection of C₉-dialdehyde. The extracted ion chromatography of substrate (apo-10′-zeaxanthinal) and its products I (zaxinone) and II (C₉-dialdehyde) in vitro catalyzed by ZAS (top). Derivatization reaction of C₉-dialdehyde is shown below the chromatogram (bottom).

However, the C₉-dialdehyde was also detected in the negative control (the void plasmid) sample, which could be due to non-enzymatic (non-specific) oxidation. For further confirmation of ZAS activity, the amount of C₉-dialdehyde was quantified in both samples.
The substrate decreased 1.56 times in ZAS samples compared to the control. The quantity of the C_{18} product, zaxinone, was 63 times more in the ZAS samples than in the control ones where it was only present in traces. Similarly, the amount of the C_{9} dialdehyde was 39 times higher in the ZAS sample compared to the control (Figure 26).

![Figure 26](image)

Figure 26. (A) The relative abundance of ZAS substrate and products. The peak areas of ZAS substrate, apo-10'-zeaxanthinal; (B) the 2 products; zaxinone and (C) the derivatized C_{9} dialdehyde were calculated with respect to the internal standard peak area. Quantification was done after an hour of incubation; data is average of three replicates.

3.4.5. *In vitro* assay with purified ZAS protein

To confirm the activity observed with crude lysate of overexpressing *E. coli* cells, we purified the enzyme and conducted corresponding assays. For this purpose, we expressed ZAS coding sequences in *E. coli* fused with *maltose-binding protein (MBP)* gene and purified MBP-ZAS
(Figure 27 A). Then, we incubated purified MBP-ZAS with apo-10'-zeaxanthinal. HPLC analysis showed that, like crude protein, purified ZAS protein is active when apo-10'-zeaxanthinal was used as a substrate (Figure 27B). Similarly, purified ZAS converts almost all the substrate, apo-10'-zeaxanthinal to zaxinone. These results confirmed the cleavage activity as well as the substrate specificity of ZAS.

Figure 27. UHPLC analysis of purified ZAS incubation with apo-10'-zeaxanthinal. (A) Coomassie Blue-stained SDS/PAGE gel analysis of maltose binding protein (MBP)-ZAS purification fractions. Lanes represent: M, prestained protein molecular weight marker (size in kDa); total protein extract of control cells, an aliquot corresponding to 100 µl culture, before A and after induction with 0.1 mM IPTG B, total protein extract of cells, an aliquot corresponding to 100 µl culture, producing MBP-ZAS before C and after induction D; E, fraction of total soluble protein of MBP-ZAS producing cells, an aliquot corresponding to 150 µl culture; F, flow through fraction of E after binding to amylose, an aliquot corresponding to 150 µl culture; G, wash fraction of MBP-beads-amylose resin; H, eluate from amylose resin of ZAS-fusion protein, an aliquot corresponding to 750 µl culture; arrow indicates MBP-ZAS fusion protein. (B) UHPLC analysis of extracts obtained from incubation with crude lysate of MBP-ZAS producing cells, purified MBP-ZAS. Control assays were performed with crude lysate of MBP expressing cells. The incubation with apo-10'-zeaxanthinal (I) led to zaxinone (II). The UV-Vis spectra of substrate (I) and product (II) are shown in the insets, mAU, arbitrary units.
3.4.6. Identification of endogenous zaxinone in rice, tobacco, and Arabidopsis

Zaxinone was identified as an *in vitro* enzymatic product. To confirm it is being a natural plant metabolite, we developed a protocol for extraction and UHPLC-MS/MS analysis of zaxinone from plant material, which allowed us to detect its presence *in planta*. Guided by our *in vitro* data which showed that zaxinone is the product of ZAS and ZAS is a rice carotenoids cleavage enzyme, first, we investigated zaxinone content in rice. UHPLC-MS/MS analysis showed uniformity in the retention time and the accurate mass of zaxinone extracted from plant tissue and the synthetic standard zaxinone. This data confirmed that zaxinone is a natural metabolite present in rice (Figure 28) as well as in tobacco and Arabidopsis (Figure 29).

![Diagram](image)

**Figure 28.** Identification of endogenous zaxinone in rice based on retention time (Left), accurate mass and MS/MS pattern (Right), in comparison to authentic standard.
Figure 29. Identification of endogenous zaxinone. (A) Extracted ion chromatography; (B) full-scan spectra of fragment ions of zaxinone standard (upper), zaxinone extracted from tobacco (middle) and zaxinone extracted from Arabidopsis (bottom) are shown.
4. Discussions

SLs were formerly recognized as the signal that triggers seed germination in root parasitic weeds (Cook et al., 1966; Xie et al., 2010; Yoneyama et al., 2010) and stimulates the symbiotic relationship between plants and mycorrhizal fungi (Akiyama et al., 2005). SLs received significant attention upon the discovery of their role as plant hormones that regulate tillering/shoot-branching (Gomez-Roldan et al., 2008; Umehara et al., 2008) in response to adverse stress conditions, especially phosphate starvation (Kohlen et al., 2011; Umehara et al., 2010). There are 25 known SLs which differ in stereochemistry, ring structure and derivatization and can be classified according to their variable moiety into canonical, tricyclic lactone (ABC ring) - containing SLs and non-canonical SLs, characterized by a conserved D-rind but lack the tricyclic structure (Al-Babili and Bouwmeester, 2015). Recent researches expanded our knowledge about SLs functions and revealed their involvement in the regulation of root architecture and other phases of plant development (Al-Babili and Bouwmeester, 2015).

Several studies contributed to the elucidation of SL biosynthesis. This was achieved by an in-depth characterization of the enzymes involved in the synthesis of SL from β-carotene and the corresponding mutants in various plant species; D27 (Alder et al., 2012; Lin et al., 2009; Waters et al., 2012), CCD7 (Alder et al., 2012; Booker et al., 2004; Drummond et al., 2009; Zou et al., 2006) and CCD8 (Alder et al., 2012; Arite et al., 2007; Simons et al., 2007; Snowden et al., 2005; Sorefan et al., 2003). Additionally, the enzymes that convert CL to various SLs, are MAX1s and LBO (Abe et al., 2014; Alder et al., 2012; Brewer et al., 2016; Zhang et al., 2014). The present thesis investigates the biosynthesis of new plant growth regulators and signaling molecules, which could be structurally derived from β-carotene or other carotenoids, and provide evidence for their biological significance.
4.1. 3-H-CL, a novel non-canonical SL

We identified a new CL-like metabolite, 3-H-CL, as a product of the SL core pathway. We speculated that 3-H-CL is formed in a parallel path to the one leading to SLs via CL. In addition, we assume 3-H-CL is the starting point of a new branch in SL biosynthesis and the precursor of yet unidentified SLs. In our study, the in vitro approach was employed for several reasons. First, SLs and CL are present in plants at very low concentrations. Secondly, both are difficult to detect and to identify without a standard. Moreover, the chemical synthesis is complicated and the yield is very low, and most importantly, they are unstable and can be easily degraded or isomerized.

Our in vitro results showed the formation of 3-H-CL from 9-cis-3-OH-β-apo-10′-carotenal by the action of CCD8. This 9-cis-3-OH-β-apo-10′-carotenal is the direct product of 9-cis-zeaxanthin cleavage by CCD7. Yet, the enzymatic steps leading to the formation of 9-cis-zeaxanthin from all-trans-β-carotene are still not clear.

Other potential producers of CLs that we examined are carotenoids other than β-carotene, supported by that fact that CCD7 cleaves 9-cis-substrates derived from α-carotene, which might provide alternative substrates for CCD8 (Bruno et al., 2014). This idea was also inspired by the discovery of heliolactone, which was identified as a CL-like molecule and is structurally derived from α-carotene (Ueno et al., 2014). We investigated the possibility of having α-carotene-derived CL-like molecules that can be produced by the action of CCD8 on α-carotene-derived apocarotenals. However, no product was formed upon in vitro incubations of CCD8 with 9-cis-α-apo-10′-carotenal and 3-OH-9-cis-α-apo-10′-carotenal, which confirms that 3-H-CL is derived from β-carotene.
4.1.1. The possible role of D27 in 3-H-CL biosynthesis

SLs are synthesized via the central metabolite and biologically active compound CL that arises by the sequential action of the β-carotene isomerase D27 and the carotenoid cleavage dioxygenases (CCDs) CCD7 and CCD8 (Alder et al., 2012). In this pathway, CCD7 cleaves the D27 product 9-cis-β-carotene into 9-cis-β-apo-10’-carotenal that is further converted by CCD8 into CL (Alder et al., 2012).

D27 is an iron-binding protein that does not show homology to any known enzyme. However, several lines of evidence have revealed the role of D27 in SL biosynthesis as the first enzyme in the path from β-carotene to CL (Alder et al., 2012; Lin et al., 2009; Waters et al., 2012).

A detailed study on the substrate specificity of OsD27 (Bruno and Al-Babili, 2016) addressed two important questions. The first question, which was raised by the substrate range of CCD7, is whether or not D27 is the isomerase that provides the other 9-cis-configured substrates of CCD7. Secondly, they investigated the involvement of D27 in ABA biosynthesis as an all-trans/9-cis isomerase of violaxanthin.

It was previously shown that D27 catalyzes the reversible isomerization of all-trans- into 9-cis-β-carotene (Alder et al., 2012) Furthermore, it was demonstrated that rice D27 (OsD27) is not only stereo-specific, but also has a strict double bond-specificity as it targets the C9, C10 double bond (Bruno and Al-Babili, 2016). OsD27 isomerizes bicyclic carotenoid substrates with at least one β-ionone ring which is not substituted, such as α-carotene and β, β-cryptoxanthin, in addition to the established substrate β-carotene. Therefore, OsD27 did not convert all-trans- violaxanthin to 9-cis-violaxanthin, the precursor of ABA, which eliminates the direct involvement of D27 in ABA biosynthesis. Moreover, it was shown that D27 is incapable of isomerizing zeaxanthin in vitro (Bruno and Al-Babili, 2016).
To shed some light on a possible role of OsD27 in the biosynthesis of 3-H-CL in planta, we used the transient expression approach in tobacco leaves. Recently, it was shown that CL is produced in *Nicotiana benthamiana* leaves upon overexpression of the rice and tomato SL biosynthetic genes *D27*, *CCD7*, and *CCD8* (Zhang et al., 2014; 2018). Here, we showed that 3-H-CL could also be formed in planta when rice and Arabidopsis SL biosynthetic genes *D27*, *CCD7* and *CCD8* were transiently expressed in *Nicotiana benthamiana* leaves. This suggests that all three enzymes D27, CCD7 and CCD8, are required for the biosynthesis of 3-H-CL.

Furthermore, we speculate that the precursor of 3-H-CL, 9-cis-3-OH-β-apo-10’-carotenal, is produced from 9-cis-zeaxanthin that can arise directly from 9-cis-β-carotene by hydroxylation of the β-ionone rings (Figure 30B). This assumption is based on the narrow substrate specificity of OsD27 which was shown by. It is unlikely that all-trans/9-cis isomerization of zeaxanthin takes place after the hydroxylation (Bruno and Al-Babili, 2016).
Figure 30. (A) The pathway to CL and 3-H-CL. D27 catalyzes the isomerization of all-trans-β-carotene (C₄₀) to 9-cis-β-carotene which is cleaved by CCD7 to 9-cis-β-apo-10′-carotenal and then converted by CCD8 to CL. (B) The proposed pathway to 3-H-CL. CCD8 converts 9-cis-3-OH-β-apo-10′-carotenal to 3-H-CL. We assumed that CCD7 provides the hydroxylated substrate to CCD8. The synthesis of the precursor, 9-cis-zeaxanthin from all-trans-β-carotene requires hydroxylation and isomerization; the order of these two steps is still unknown.

4.1.2. Is 3-H-CL a natural metabolite?

Recent studies suggest that some uncharacterized apocarotenoids, apart from ABA and SLs, can regulate plant development and biological processes including stress response (Alagoz et al., 2018; Hou et al., 2016; Seto and Yamaguchi, 2014). Therefore, there is a hunt to identify novel apocarotenoid signaling molecules and to reveal their role in various aspects of plant life (Hou et al., 2016; McQuinn et al., 2015). CL was identified as an endogenous precursor of SLs in rice and Arabidopsis (Seto et al., 2014). Several studies show the elevated levels of CL in the d14 mutant of rice and Arabidopsis in comparison with wild-type plants. Similarly, we checked the presence of endogenous 3-H-CL in rice by analyzing the roots of the d14 mutant. As expected,
we detected 3-H-CL by using UHPLC-MS/MS in rice roots. However, we could not detect 3-H-CL in the Arabidopsis Atld14 mutant; possibly because Arabidopsis produces a considerably low amount of 3-H-CL, compared to rice (Seto et al., 2014). Taken together, our results demonstrate that 3-H-CL is an endogenous carotenoid-derived intermediate that is likely a biosynthetic precursor of new SLs.

4.3. ZAS, a member of new CCD sub-family

Carotenoid cleavage dioxygenases (CCDs) are a family of enzymes that catalyze the cleavage of C-C double bonds in carotenoid substrates at various positions resulting in different apocarotenoids. Over the years, several new CCDs have been identified based on homology with the first identified CCD, VP14 from maize (Ahrazem et al., 2016; Schwartz et al., 1997; Sui et al., 2013). In this part of the thesis, we identified a novel CCD that shows sequence homology to CCD8. We predicted that the LOC Os09g15240-encoded enzyme, Zaxinone Synthase (ZAS) would have a cleavage site (C13-C14) similar to CCD8. CCD8 catalyzes the conversion of 9-cis-β-apo-10’-carotenal to CL in a 9-cis pathway. We showed that CCD8 can also convert 9-cis-3-OH-β-apo-10’-carotenal into the hydroxylated CL. In addition to these two 9-cis-cleavage reactions, CCD8 catalyzes a standard CCD cleavage reaction with the all-trans-β-apo-10’-carotenal, at C13-C14, to produce β-apo-13-apocarotenone (Alder et al., 2008; Schwartz et al., 2004) which involves deoxygenation via the intermediate dioxygenate (Figure 3). The product of the all-trans reaction “D’orenone” (β-apo-13-carotenone) is the non-hydroxylated form of zaxinone (Figure 31). All three described reactions of CCD8 involve bond cleavage at the C13, C14 position (Alder et al., 2012; Bruno et al., 2017). Our in vitro investigations of ZAS activity showed that it cleaves several all-trans-apocarotenoids at the C13-C14 position, like CCD8. These findings revealed the regio-specificity of ZAS.
The ZAS cleavage reaction yielded the primary cleavage product, zaxinone (C_{18}), and a second smaller product, a C_{9} dialdehyde. To confirm the single cleavage of the C_{27} substrate, apo-10’-zeaxanthinal, at the C_{13}, C_{14} double bond, it is essential to identify the second product which should be a C_{9} dialdehyde. However, we were not able to detect the second product by HPLC or LC-MS due to its small size, instability, and high reactivity. The short chain dialdehyde is difficult to identify because it reacts with secondary amines, amino acids such as lysine residues to form a Schiff base. Therefore, the C_{9} dialdehyde needs to be derivatized in order to stabilize it and allow its detection by LC-MS. O-(2,3,4,5,6 Pentafluorobenzyl) hydroxylamine hydrochloride is the reagent we used to conjugate the dialdehyde from both sides to overcome its instability, by forming a Shiff base (Figure 9). Conjugating the second product with C_{23}H_{18}O_{2}N_{2}F_{10} enabled its detection by LC-MS in the samples after in vitro incubation of the substrate apo-10’-zeaxanthinal with ZAS. This confirmed the single cleavage function of ZAS as well as the specific cleavage position.
Figure 31. The C13-C14 double bond cleavage by CCD8 and ZAS. (A) The C13-C14 double bond cleavage by CCD8 of all-trans-β-apo-10’-carotenal yields two carbonyl products; β-apo-13-carotenone and C₉ dialdehyde that arise concomitantly with it. (B) The C13, C14 double bond cleavage of all-trans-3-OH-β-apo-10’-carotenal (apo-10’-zeaxanthinal) produces zaxinone and a secondary product, C₉ dialdehyde.

For quantification of the ZAS substrate and its products, the formation of the product was monitored by calculating the ratio between the peak areas of derivatized C₉ in the control and ZAS samples. For proper and accurate quantification, an internal standard is needed to correct the values and account for the losses during the sample handling. A customized synthetic C₁₀ dialdehyde was used as the internal standard. For further confirmation, the evolution of zaxinone (C₁₈) and the decrease in the apo-10’-zeaxanthinal (C₂₇) were also monitored. We found that the concentration of C₉ dialdehyde was increased 39-fold in the ZAS sample compared to the control, which confirmed that it is the cleavage product produced by the action of ZAS. This concludes that the enzyme (ZAS) performs a single cleavage specifically at the C13, C14 double bond to form zaxinone and the C₉ dialdehyde.
The HPLC analysis of the in vitro assays showed that besides the substrate apo-10’-zeaxanthinal, ZAS also cleaved stereo- and regio- selectively other all-trans-configured hydroxylated apocarotenoids, i.e. 3-OH-β-apo-8’-carotenal (apo-8’-zeaxanthinal) and 3-OH-β-apo-12’-carotenal (apo-12’-zeaxanthinal), yielding zaxinone. However, the activity with the other substrates was comparatively lower. We studied the substrate preference of ZAS by performing a conversion rate study. By choosing the best substrates, apo-10’-zeaxanthinal (C_{27}) and apo-8’-zeaxanthinal (C_{30}) and comparing the product evolution over time with each substrate, we demonstrated that ZAS has higher conversion rates with apo-10’-zeaxanthinal compared to apo-8’-zeaxanthinal, indicating that the former is the preferred substrate.

4.4. Zaxinone, a novel regulatory molecule

In addition to recent characterized apocarotenoid-derived regulatory molecules, there are still unidentified molecules that can be classified as bioactive plant apocarotenoids. Several studies on carotenoid biosynthesis inhibitors along with the investigations of different plant mutants indicated the possible role of yet uncharacterized carotenoid-derived molecules in plant growth and development (Al-Babili, 2017). These molecules can be involved in the regulation of processes such as carotenoids biosynthesis (Kachanovsky et al., 2012), lateral root formation and chloroplast and leaf development (Avendaño-Vázquez et al., 2014).

Here, we identified a new plant apocarotenoid molecule, zaxinone, which increases root biomass in rice. Our work demonstrates that zaxinone synthesis requires the activity of the ZAS. My colleagues in The Bioactives LAB (KAUST) studied the biological importance of zaxinone via loss-of-function of the rice zas mutant (Figure 32). The zas mutant has reduced crown root length and number as well as root biomass and this phenotype was clearly rescued by exogenous application of 2.5 mM zaxinone for three weeks in a hydroponic system (Figure 32).
The zas mutant showed reduced plant height in plants grown in the greenhouse (Figure 32A), compared to the wild-type. In addition, main crown root length, number of crown roots, and root and shoot biomass observed after three weeks were considerably reduced in zas (Figure 32B). Interestingly, application of zaxinone (2.5 µM) to zas mutant rescued the roots phenotype; i.e. crown root length, number of crown roots and root biomass in zas became similar to those of wild-type (Figure 32B).

These data show that zaxinone acts as a positive regulator of growth in rice. Remarkably, this phenotype in zas mutant was accompanied by a higher SL, 4-deoxyorobanchol content in root tissues and exudates, and higher Striga seed germination, compared to wild-type (data not shown). Higher SL content in zas was further aligned with higher transcript levels of SL biosynthesis genes D27, CCD7, CCD8 and carlactone oxidase (CO) (data not shown). Following, 5 µM zaxinone treatment for 6 hours, reduced the SL content and Striga seed germination in Pi-starved zas seedlings. Also, transcript levels of SL biosynthesis genes were decreased in the zas mutant (data not shown). The data demonstrate that zaxinone negatively regulates SL content via regulation of SL biosynthesis in phosphate-starved conditions.

Overall, zaxinone is a novel carotenoid-derived regulatory metabolite, which positively regulates the growth and development of rice. In addition, zaxinone may be a useful molecule to fight against witchweed, Striga.
Figure 32. Phenotypic characterization and rescue of zas mutant. (A) The phenotype of Nipponbare wild-type (WT) and zas mutant at heading stage. Data are means ± SD, n = 10. (B) Rescued of crown root length and number in zas by 2.5 µM zaxinone in the hydroponic system. Data are means ± SD, n = 6. Mock treatment (0.1 % acetone). Different letters above each bar denote significant difference following a One-way ANOVA test (P < 0.05) (Wang et al., unpublished).
5. Summary

Strigolactones (SLs) are a new class of plant hormones that are important for establishing normal plant architecture and for plant’s response to different types of stress. For the past 60 years, SLs have been known as germination stimulants for parasitic weeds. More recently, they have been found to mediate the symbiotic interaction between plants and arbuscular mycorrhizal (AM) fungi. The biosynthesis of these carotenoid-derived compounds was elucidated later on guided by the investigation of mutants from different plant species, which showed the involvement of two carotenoid cleavage dioxygenases (CCD7 and CCD8), a β-carotene isomerase DWARF27 (D27) and a cytochrome P450 enzyme more axillary growth1 (MAX1).

Several studies proposed the existence of some uncharacterized apocarotenoids involved in the regulation of plant development and their stress response, which explain the growing interest to search for new carotenoids-derived signaling metabolites and to study their effect on plant.

The enzymatic study and the bioassays we performed in this work enabled the discovery of two novel carotenoid-derived metabolites present in planta, 3-OH-carlactone (3-H-CL) and zaxinone.

Our in vitro enzymatic assay showed that CCD8 can convert 9-cis-3-OH-β-apo-10’-carotenal to hydroxylated carlactone (3-H-CL) in a parallel path to the formation CL, which utilizes the same enzymes. We identified 3-H-CL, a possible intermediate for hydroxylated SLs. Next, we verified the functionality of this pathway by transient expression of the three enzymes D27, CCD7 and CCD8 in tobacco leaves, which are involved in both CL and 3-H-CL biosynthesis, from rice and Arabidopsis. Co-expression of all three enzymes led to the accumulation of 3-H-CL in planta. The structure of this new product of CCD8 was confirmed to be 3-H-CL by NMR analysis and in comparison to a synthetic standard. We also revealed that 3-H-CL is an endogenous metabolite by confirming its presence in the roots of the rice SL perception mutant d14. Further, we
investigated the biological significance of 3-H-CL by testing its SL-like functions. We showed that 3-H-CL is a biologically active compound, inducing seed germination of root parasitic weed *Striga hermonthica*, through a parasitic seed germination bioassay. We also studied the effect of 3-H-CL on shoot architecture and found that feeding rice *d10* with 3-H-CL restored the wild-type tillering phenotype, which raises the possibility of 3-H-CL having hormonal activity.

Carotenoid cleavage dioxygenases (CCDs) are responsible for specific oxidative cleavage of carotenoids to apocarotenoids and are divided into five subgroups, including the above-mentioned CCD7 and CCD8 groups which are involved in SL biosynthesis. A survey on CCDs of maize, sorghum, and rice revealed a clade missing in Arabidopsis. We analyzed the distribution of this subgroup in the plant kingdom and investigated the enzymatic activity of a rice representative encoded by *LOC_Os09g15240*, which we identified as zaxinone synthase (ZAS). The enzymatic studies on this particular enzyme with different substrates performed in this thesis aimed at elucidating its cleavage pattern and substrate specificity. Our enzymatic study of ZAS showed that it does not cleave intact carotenoids, but several all-trans-configured, hydroxylated apocarotenoids with varying chain lengths, and that apo-10’-zeaxanthinal is the best substrate. ZAS performs a cleavage at the C13, C14 double bond of the C27 substrate apo-10’-zeaxanthinal, which results in two products, a C18 ketone that we named zaxinone and unstable C9 dialdehyde identified as apo-14, 10-carotene-dial. Moreover, the studying of the *zas* mutant and of biological effects of exogenous zaxinone treatment shows that zaxinone is a novel carotenoid-derived signaling molecule that is essential for normal rice development.
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SUPPLEMENTAL DATA

Materials and Methods

NMR analysis

For NMR analysis, approximately 1 mg purified 3-H-CL was dissolved in 40 μL of CD₂Cl₂. The NMR experiments were performed on a Bruker AVANCE II spectrometer operating at a ¹H resonance frequency of 500.13 MHz. The instrument is equipped with a 1.7 TXI triple (¹H, ¹³C, ³¹P) microprobe with a z-gradient coil and with pulse angles of 5.9 μs (¹H) and 14.9 μs (¹³C), respectively. Bruker TOPSPIN software (version 3.2, patch level 3) was used to acquire and process the NMR data. All measurements were carried out at a temperature of 300 K.

For the 1D ¹H experiment, the parameters were as follows: standard one-pulse experiment (zg), 32 transients, a spectral width of 12 ppm, a data size of 32K points, a relaxation delay of 6 s, an acquisition time of 2.73 s were used. For 2D ¹H-¹H-COSY the gradient-enhanced COSY experiment for magnitude mode of detection (cosygpppf), 32 scans, a relaxation delay of 1s, an acquisition time of 0.17 s, a spectral width in both dimensions (f2, f1) of 12 ppm, 2k and 128 acquired data points in f2 and f1, respectively were used. Data were processed by zero filling (to 1024) in f1 and by using unshifted squared sine window functions in both dimensions prior to 2D Fourier transformation. For carbon assignments, 2D ¹H-¹³C-HSQC and 2D ¹H-¹³C-HMBC were acquired (Supplemental Figures 1 to 8).

Chemical synthesis of 3-H-CL

3-H-CL (1) was synthesized as shown in Scheme (Supplemental Figure 11) starting from 4-hydroxy-2, 2, 6-trimethylcyclohexan-1-one (2) that was obtained in two steps from 4-ketoisophorone as described previously (Abrams et al., 1990). After protection of the hydroxyl
group by an isopropyldimethylsilyl (IPDMS) group, the resultant silyl-protected cyclohexanone 3 was converted to the triflate 4, which was subjected to the Heck reaction with methyl vinyl ketone to afford 3-IPDMSO-β-ionone (5). The sily-protected ionone 5 was converted to IPDMSO-C14-aldehyde 7 via the Corey–Chaykovsky epoxidation, followed by methylaluminium bis (4-bromo-2,6-di-tert-butylphenoxide) (MABR)-promoted rearrangement of epoxide to aldehyde. The IPDMS group of 7 was removed by C18 reversed-phase silica gel-mediated hydrolysis, yielding the desired hydroxy-aldehyde 8 (Mori et al., 2016). O-Alkylation of the potassium enolate of aldehyde 8 with bromobutenolide 9 in the presence of 18-crown-6-ether furnished 3-H-CL (1) and its (9E) geometric isomer in a ratio 1:5. Purification of semi-preparative C18 reversed-phase HPLC afforded 3-H-CL (1) as a mixture of two epimers at C-11 that was not separable under the conditions used.

Mass spectra were recorded on a JMS-700 instrument (JEOL). 1H- and 13C-NMR spectra were obtained with a JNM-AL400 NMR spectrometer (JEOL). Chemical shifts were referenced to tetramethylsilane as an internal standard. Column chromatography was performed with Wakogel C-200 (Wako Pure Chemical Ind.), Kieselgel 60 (Merck), Chromatorex ODS (Fuji Silysia Chemical), and Inertsil ODS-3 (ϕ 10 × 250 mm, 5 μM; GL Sciences).

4-((isopropylidimethylsilyl)oxy)-2,2,6-trimethylcyclohexan-1-one (3)

A mixture of hydroxy-cyclohexanone 2 (3.88 g, 24.8 mmol), isopropylidimethylsilylchlorosilane (7.87 mL, 50.7 mmol), and imidazole (6.75 g, 99.2 mmol) in N,N-dimethylformamide (DMF, 63 mL) was stirred at room temperature for 3 h under argon. The reaction mixture was taken up in 2% (v/v) ether in n-hexane, washed with water, dried over anhydrous Na2SO4, and concentrated in vacuo. Purification by silica gel column chromatography
eluted stepwise with n-hexane and ether [2% (v/v) increments] gave isopropylidemethylsilyloxy-cyclohexanone 3 (8.60 g, 33.6 mmol, quant.). $^1$H-NMR (CDCl$_3$, 400 MHz) δ: 0.09 (6H, s, Si-(CH$_3$)$_2$), 0.79-0.88 (1H, m, Si-CH), 0.99 (6H, d, J = 7.8 Hz, Si-CH-(CH$_3$)$_2$), 1.00 (3H, s, 2-CH$_3$), 1.01 (3H, d, J = 7.2 Hz, 6-CH$_3$), 1.34 (3H, s, 2-CH$_3$), 1.57-2.04 (4H, m, H-3 and -5), 3.14-3.23 (1H, m, H-6), 4.09-4.12 (1H, m, H-4); $^{13}$C-NMR (CDCl$_3$, 100MHz) δ: −3.9, −3.8, 14.6, 14.7, 16.9, 26.4, 28.5, 35.3, 43.8, 47.9, 66.4, 218.1; EI-MS m/z: 256 [M]+, 199; HREIMS m/z: 256.1833 [M]$^+$ (calcd. for C$_{14}$H$_{28}$O$_2$, m/z 256.1859).

4-((isopropylidemethylsilyl)oxy)-2,6,6-trimethylcyclohex-1-en-1-yl trifluoromethanesulfonate (4)

To a solution of lithium diisopropylamide (0.42 M in THF and hexane, 88 mL, 37 mmol) was added isopropylidemethylsilyloxy-cyclohexanone 3 (8.60 g, 33.6 mmol) in THF (46 mL) dropwise at −78 °C under argon. The mixture was stirred for 1 h and then N-phenylbis(trifluoromethanesulfonimide) (12.6 g, 35.3 mmol) in THF (46 mL) was added. The mixture was stirred for 1 h and then allow to warm to room temperature, and stirred for 15 h. The reaction mixture was poured into saturated aqueous NH$_4$Cl, and the organic layer was separated and the water layer was extracted three times with ether. The organic phase was washed with brine, dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. Purification by silica gel column chromatography eluting with n-hexane gave the triflate 4 (12.7 g, 32.6 mmol, 97%). $^1$H-NMR (CDCl$_3$, 400 MHz) δ: 0.08 (6H, s, Si-(CH$_3$)$_2$), 0.80-0.90 (1H, m, Si-CH), 0.97 (6H, d, J = 6.8 Hz, Si-CH-(CH$_3$)$_2$), 1.14 (3H, s, 6-CH$_3$), 1.21 (3H, s, 6-CH$_3$), 1.62-2.38 (4H, m, H-3 and -5), 1.75 (3H, s, 2-CH$_3$), 3.97-4.05 (1H, m, H-4); $^{13}$C-NMR (CDCl$_3$, 100MHz) δ: −3.9, −3.8, 14.7, 16.9, 26.5, 27.3, 36.7, 41.5, 49.2, 64.0, 118.7 (d, J = 318.9 Hz), 123.9, 148.9; EI-MS m/z: [M]$^+$ was not observed, 239, 121.
(E)-4-(4-((isopropyldimethylsilyl)oxy)-2,6,6-trimethylcyclohex-1-en-1-yl)but-3-en-2-one (5)

A mixture of triflate 4 (12.7 g, 32.6 mmol), triethylamine (18.1 mL, 130 mmol), methyl vinyl ketone (5.31 mL, 65.2 mmol), and bis(triphenylphosphine) palladium(II) dichloride (432 mg, 0.2 mmol) in DMF (85 mL) was stirred at 75 °C for 22 h under argon. The reaction mixture was ice-cooled, quenched by adding water, and extracted with n-hexane. The organic phase was washed with water, dried over anhydrous Na₂SO₄, and concentrated in vacuo. Purification by silica gel column chromatography using 3% (v/v) stepwise elution with ether and n-hexane gave the ionone 5 (3.60 g, 11.7 mmol, 36%).

1H-NMR (CDCl₃, 400 MHz) δ: 0.089 (3H, s, Si-CH₃), 0.091 (3H, s, Si-CH₃), 0.80-0.90 (1H, m, Si-CH), 0.97 (6H, d, J = 6.8 Hz, Si-CH-(CH₃)₂), 1.01 (3H, s, 6'-CH₃), 1.03 (3H, s, 6'-CH₃), 1.42-2.23 (4H, m, H-3' and -5'), 1.76 (3H, s, 2'-CH₃), 2.30 (3H, s, 1'-CH₃), 3.88-3.96 (1H, m, H-4'), 6.11 (1H, d, J = 16.4 Hz, H-1'), 7.22 (1H, d, J = 16.1 Hz, H-2'); 13C-NMR (CDCl₃, 100MHz) δ: −3.8, 14.7, 16.9, 21.6, 27.2, 28.4, 30, 36.8, 43.3, 48.8, 64.9, 132.1, 133.1, 135.4, 142.4, 198.6; EI-MS m/z: 308 [M]+, 293, 265, 147, 121; HREIMS m/z: 308.2163 [M]+ (calcd. for C₁₈H₃₂O₂Si, m/z 308.2172).

(E)-isopropylidimethyl((3,5,5-trimethyl-4-(2-(2-methyloxiran-2-yl)vinyl)cyclohex-3-en-1-yl)oxy)silane (6)

To a solution of trimethylsulfonium iodide (3.60 g, 17.5 mmol) in dimethyl sulfoxide (DMSO, 14.8) was added THF (14.8 mL) under argon to yield a finely divided suspension of sulfonium salt. This mixture was then cooled to −5 °C and treated with a solution of dimsyl sodium (4.4 M, 4.54 mL, 20.0 mmol). The resulting gray colored suspension was treated with a solution of 3-isopropylidimethylsilyloxy-β-ionone 5 (3.60 g, 11.7 mmol) in THF (3.6 mL). After stirring at −5 °C for 45 min, the mixture was warmed to room temperature, quenched by successively adding water (75 mL) and n-hexane (75 mL). The organic phase was washed with
water, dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo to give the epoxide 6. (3.66 g, 11.4 mmol, quant.). $^1$H-NMR (CDCl$_3$, 400 MHz) δ: 0.08 (6H, s, Si-(CH$_3$)$_2$), 0.80-0.90 (1H, m, Si-CH), 0.97 (6H, d, $J = 6.8$ Hz, Si-CH-(CH$_3$)$_2$), 1.01 (3H, s, 6''-CH$_3$), 1.03 (3H, s, 6''-CH$_3$), 1.42-2.23 (4H, m, H-3'' and -5''), 1.51 (3H, s, 2- CH$_3$), 1.66 (3H, s, 2''-CH$_3$), 2.75 (1H, d, $J = 5.6$ Hz, H-3α), 2.84 (1H, d, $J = 5.6$ Hz, H-3β), 3.88-3.96 (1H, m, H-4''), 5.25 and 5.27 (1H, d, $J = 16.1$ Hz, H-1'), 6.136 and 6.138 (1H, d, $J = 16.1$ Hz, H-2''); $^{13}$C-NMR (CDCl$_3$, 100MHz) δ: −3.7, 14.8, 17.0, 21.22 and 21.23, 28.3, 29.99 and 30.1, 36.8, 42.7, 48.5, 65.4-3.7, 14.2, 14.8, 17.0, 19.66 and 19.74, 21.0, 21.22 and 21.24, 28.3, 36.8, 42.7, 48.5, 55.76 and 55.79, 55.9, 56.0, 60.4, 65.4, 126.75 and 126.79, 129.31 and 129.42, 134.82 and 134.88, 136.17 and 136.23; EI-MS m/z: 322 [M]+, 265, 163, 121; HREIMS m/z: 322.2315 [M]+ (calcd. for C$_{19}$H$_{34}$O$_2$Si, m/z 322.2328).

(E)-4-(4-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl)-2-methylbut-3-enal (8)

To a solution of 2,6-di-tert-butyl-4-bromophenol (13.0 g, 46.7 mmol) in dichloromethane (100 mL) was added at room temperature a 1.4 M hexane solution of trimethylaluminium (Me$_3$Al, 16 mL, 22.4 mmol), and the solution was stirred at room temperature for 1 h under argon. To a solution of the MABR (22 mmol) in dichloromethane was added a solution of epoxide 6 (3.67 g, 11.4 mmol) in dichloromethane (7.7 mL) at −78 °C, and the resulting mixture was stirred at −78 °C for 30 min under argon. The reaction mixture was poured into water, and extracted with $n$-hexane. The organic phase was washed with saturated aqueous NaHCO$_3$, dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. The residue containing the silyl protected aldehyde 7 was dissolved in 70% (v/v) acetonitrile in water and applied to ODS column, left to stand for 14 h, and then eluted stepwise with 70–100% (v/v) acetonitrile in water [5% (v/v) increments]; 75–80% acetonitrile eluates were combined, evaporated to water in vacuo, extracted with $n$-hexane, and concentrated in vacuo to give the aldehyde 8 (208 mg, 2.6%). $^1$H-NMR
(CDCl₃, 400 MHz) δ: 1.03 (6H, s, 6'-CH₃), 1.24 and 1.26 (3H, d, J = 1.5 Hz, 2-CH₃), 1.45–2.36 (4H, m, H-3' and -5'), 1.69 (3H, s, 2'-CH₃), 3.13-3.19 (1H, m, H-2), 3.97-4.00 (1H, m, H-4'), 5.34 (1H, dd, J = 16.0, 7.4 Hz, H-3), 5.99 (1H, d, J = 16.0 Hz, H-4), 9.62-9.63 (1H, m, H-1);

¹³C-NMR (CDCl₃, 100 MHz) δ: 13.6, 14.2, 15.3, 16.7, 21.27 and 21.29, 42.0, 48.0, 50.7, 60.4, 126.2, 130.2 and 130.3, 130.97 and 130.99, 136.8, 201.7; EI-MS m/z: 222 [M]+, 193, 175; HREIMS m/z: 222.1602 [M]+ (calcd. for C₁₄H₂₂O₂ m/z 222.1620).

5-(((1Z,3E)-4-(4-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl)-2-methylbuta-1,3-dien-1-yl)oxy)-3-methylfuran-2(5H)-one (3-HO-CL) (1)

To a mixture of 3-HO-C₁₄-aldehyde 8 (965 mg, 4.35 mmol), phenothiazine (22 mg), (±)-4-bromo-2-methyl-2-buten-4-oxide 9 (513 μl, 5.22 mmol), and 18-crown-6-ether (1.26 g, 8.70 mmol) in THF (55 mL), potassium tert-butoxide (976 mg, 8.70 mmol) was added slowly under argon, and the reaction mixture was stirred at room temperature under argon. After stirring for 45 min, the mixture was poured into cold brine (100 mL) and extracted with ether. The organic phase was washed with brine, dried over anhydrous Na₂SO₄, and concentrated in vacuo. The residue was subjected to silica gel column chromatography eluted stepwise with n-hexane and EtOAc [5% (v/v) increments]. The 40-50% EtOAc eluates containing 3-HO-CL (3-H-CL) was purified by a semi-preparative Inertsil ODS-3 HPLC (ϕ10 × 250 mm, 5 μm; GL Sciences), using isocratic elution with 60% acetonitrile in water at a flow rate of 4.0 mL/min and monitored at 254 nm to give 3-HO-CL 1 [13.4 mg, 42.1 μmol, 0.97%, retention time 5.7 min]. 3-HO-CL (as a mixture with 11-epimer): ¹H-NMR (CD₂Cl₂, 400 MHz); 1.03 (3H, s, CH₃-16 or 17), 1.04 (3H, s, CH₃-16 or 17), 1.42 (1H, m, H-2α), 1.70 (3H, s, CH₃-18), 1.74 (1H, m, H-2β), 1.92 (3H, d, J = 1.5 Hz, CH₃-19), 1.92 (3H, t, J = 1.5 Hz, H-15), 1.97 (1H, m, H-4α), 2.33 (1H, dd, J = 5.5, 16.5 Hz, H-4β), 3.94 (1H, m, H-3), 5.93 (1H, br p, J = 1.5 Hz, H-11), 5.99 (1H, br.d, J = 16.1 Hz, H-
\[ 7\), 6.21 (1H, br.s, H-10), 6.47 (1H, br.d, J = 16.1 Hz, H-8), 6.90 (1H, br p, J = 1.5 Hz, H-12); \]
\[ {^1}H-NMR (C_6D_6, 400 MHz) \square: 1.03, 1.060, 1.05 and 1.062 (total 6H, s, CH_3-16 or 17), 1.38 (3H, dd, J = 1.5, 2.9 Hz, CH_3-15), 1.43 (1H, dd, J = 3.90, 11.7 Hz, H-2α), 1.601 and 1.604 (3H, s, CH_3-19), 1.62-1.64 (1H, m, CH_3-18), 1.94 (1H, dt, J = 7.3, 16.9 Hz, H-4α), 2.18 (1H, dt, J = 5.9, 16.9 Hz, H-4β), 3.78 (1H, m, H-3), 5.21 (1H, m, H-11), 5.81 (1H, dq, J = 1.5, 2.0 Hz, H-12), 5.99 (1H, s, H-10), 6.08 (1H, br.d, J = 16.6 Hz, H-7), 6.87 (1H, d, J = 16.6 Hz, H-8) ; {^{13}}C-NMR (CD_2Cl_2, 100 MHz) \square: 10.7, 14.4, 21.6, 28.7, 30.3, 37.2, 42.7, 48.7, 65.1, 100.7, 117.25 and 117.28, 128.11 and 128.12, 126.4, 126.48 and 126.49, 135.02, 137.93 and 137.95, 138.57 and 138.60, 142.6, 171.5; {^{13}}C-NMR (C_6D_6, 100 MHz) \square: 10.2, 14.2 and 14.3, 21.67 and 21.69, 28.67 and 28.71, 30.3 and 30.4, 37.0, 42.66 and 42.69, 48.67 and 48.70, 64.57 and 64.61, 100.0, 116.48 and 116.51, 126.3, 126.6 and 126.7, 128.6, 134.5, 137.8 and 137.9, 139.15 and 139.17, 141.61 and 141.64, 170.5; EI-MS m/z (rel. int): 318 [M]^{+}(11), 175(100), 97(55), 91(41); HREIMS m/z: 318.1826 [M]^{+} (calcd. for C_{19}H_{26}O_{4}, m/z 318.1831).

Results

Chemical Synthesis of 3-Hydroxy-CL

3-Hydroxy-CL (1) was synthesized as shown in Scheme (Supplemental Figure 9) by starting from 4-hydroxy-2,2,6-trimethylcyclohexan-1-one (2) that was obtained in two steps from 4-ketoisophorone as described previously (Abrams et al., 1990). After protection of the hydroxyl group by an isopropylidemethylsilyl (IPDMS) group, the resultant silyl-protected cyclohexanone 3 was converted to the triflate 4, which was subjected to the Heck reaction with methyl vinyl ketone to afford 3-IPDMSO-β-ionone (5). The sily-protected ionone 5 was converted to IPDMSO-C_{14}-aldehyde 7 via the Corey–Chaykovsky epoxidation, followed by methylaluminium bis(4-bromo-2,6-di-tert-butylphenoxide) (MABR)-promoted rearrangement of epoxide to
aldehyde. The IPDMS group of 7 was removed by C\textsubscript{18} reversed-phase silica gel-mediated hydrolysis, yielding the desired hydroxy-aldehyde 8 (Mori et al., 2016). O-Alkylation of the potassium enolate of aldehyde 8 with bromobutenolide 9 in the presence of 18-crown-6-ether furnished 3-hydroxy-CL (1) and its (9\textit{E}) geometric isomer in a ratio 1:5. Purification of semi-preparative C\textsubscript{18} reversed-phase HPLC afforded 3-hydroxy-CL (1) as a mixture of two epimers at C-11 that was not separable under the conditions used.
Supplemental Figure 1. 1D $^1$H NMR spectrum of isolated 3-H-CL in CD$_2$Cl$_2$, spectral region 5.9 – 6.95 ppm. Red: resonance assignments of 3-H-CL, blue: resonance assignments of hydrolysed lactone fragment.
Supplemental Figure 2. 1D $^1$H NMR spectrum of isolated 3-H-CL in CD$_2$Cl$_2$, spectral region 3.8 – 7 ppm.
Supplemental Figure 3. 1D $^1$H NMR spectrum of isolated 3-H-CL in CD$_2$Cl$_2$, spectral region 0.7 – 2.5 ppm.
$^1H^1H$

(A) 12- 8- 10- 7- 11- 

(B) 3- 4- 2- 2- 

3-H/ 3-H/ 4-H/
Supplemental Figure 4. Overview (top) and expansions of the 2D $^1$H-$^1$H-COSY spectrum of isolated 3-H-CL showing $^1$H-$^1$H-coupling between adjacent protons.
Supplemental Figure 5. Expansions of the 2D $^1$H-$^{13}$C-HSQC spectrum (with carbon type editing) of isolated 3-H-CL showing $^{13}$C-$^{1}$H-one-bond ($^1$J$_{CH}$) couplings. Cross peaks of CH$_3$ and CH groups appear in blue, cross peaks of CH$_2$ groups appear in red.
$^1$H-$^{13}$C HMBC
Supplemental Figure 6. Expansions of the 2D $^1$H-$^{13}$C-HMBC spectrum of isolated 3-H-CL showing $^{13}$C-$^1$H-long-range ($^\nu$J$_{CH}$) couplings across two and three bonds between protons and carbons.
Supplemental Figure 7. Expansion of the 2D $^1$H-$^1$H-ROESY spectrum of isolated 3-H-CL showing NOE correlations (blue cross peaks) between protons in spatial proximity. Cross peaks with anti-phase behavior (appearing in red and blue) are COSY-type artifacts from residual scalar coupling.
$^1$H- DOSY

$^1$H-

3-
Supplemental Figure 8. $^1$H-DOSY spectra of isolated 3-H-CL. Top: Full spectral range; below: Three spectral expansions. Red trace: resonances from 3-H-CL, black traces: resonances from faster, low molecular weight component (hydrolysed lactone fragment) and slower diffusing, higher molecular weight components.
Supplemental Figure 9. Chemical synthesis of 3-H-CL.
Supplementary Figure 10. $^1$H-NMR spectrum of 3 (400 MHz, CDCl$_3$)
Supplementary Figure 11. $^{13}$C-NMR spectrum of 3 (100 MHz, CDCl$_3$)
Supplementary Figure 12. $^1$H-NMR spectrum of 4 (400 MHz, CDCl$_3$)
Supplementary Figure. $^{13}$C-NMR spectrum of 4 (100 MHz, CDCl$_3$)
Supplementary Figure 14. $^1$H-NMR spectrum of 5 (400 MHz, CDCl$_3$)
Supplementary Figure 15. $^{13}$C-NMR spectrum of 5 (100 MHz, CDCl$_3$)
Supplementary Figure 16. $^1$H-NMR spectrum of 6 (400 MHz, CDCl$_3$)
Supplementary Figure 17. $^{13}$C-NMR spectrum of 6 (100 MHz, CDCl$_3$)
Supplementary Figure 18. $^1$H-NMR spectrum of 8 (400 MHz, CDCl$_3$)
Supplementary Figure 19. $^{13}$C-NMR spectrum of 8 (100 MHz, CDCl$_3$)
Supplementary Figure 20. $^1$H-NMR spectrum of 1 (400 MHz, CD$_2$Cl$_2$)
Supplementary Figure 21. $^{13}$C-NMR spectrum of 1 (100 MHz, CD$_2$Cl$_2$)
Supplementary Figure 22. $^1$H-NMR spectrum of 1 (400 MHz, C$_6$D$_6$)
Supplementary Figure 23. $^{13}$C-NMR spectrum of compound 1 (100 MHz, $C_6D_6$)