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Ripening-delaying peptides and their applicability to steer quality development of climacteric fruits

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Ripening-delaying peptides and their applicability to steer quality development of climacteric fruits

Processes extending the shelf-life of climacteric fruits play an important role in increasing sustainability of global food supply. As consequence of uncontrolled ripening process, significant losses in quantity and quality exist at farm level and along the food chain. Ethylene is the key phytohormone triggering fruit ripening and senescence. Recent studies on the ethylene signalling pathway have shown that the signal transduction depends essentially on the interaction of the ethylene receptor (ETR) with the signal protein EIN2 (ethylene-insensitive protein 2). A novel synthetic octapeptide (NOP-1) derived from the so-called nuclear localization signal (NLS) at the C-terminal end of EIN2 inhibits the interaction between ETR and EIN2 and may slow-down fruit ripening. The main objectives of this study were to examine the ripening delay, and the mode-of-action, of the recombinant NOP-1 on tomatoes and apples. For this purpose, NOP-1 was applied as aqueous peptide solution onto the fruits´ surfaces after their harvest. Three different experiments, each one with a specific focus and its own particularities, were conducted. Dose-response and response-time experiments were done by application of solution droplets to the surfaces of green tomatoes. Further, the effect of NOP-1 was examined on apple fruits, which produce several times more ethylene than tomatoes. In addition, the potential effect of different application methods was studied. Finally, a ripening-stage dependent effect of NOP-1, and the impact of repeated applications, were analyzed. In order to enable a time-resolved monitoring of fruit ripening, sensor-based evaluations were performed for quality assessments and compared to classic destructive quality determinations. In summary, the following results were obtained:

1. NOP-1 concentration of 1000 µMol delayed ripening of tomatoes for several days, when applied at maturity stage ‘mature green’, whereas concentrations of 400 µMol and 2000 µMol were not effective. Colour development, chlorophyll degradation and accumulation of lycopene and ß-carotene were inhibited whereas firmness was not affected. Likewise, no quality impairment was observed.

2. On apples, NOP-1 (1000 µMol) inhibited colour development and chlorophyll degradation during shelf-life. These effects were more pronounced with the brush application (surface film) than with the application of microdroplets (mimicking a spray formulation). NOP-1 did not alter ethylene release or respiration rate, whereas 1-MCP strongly suppressed both. NOP-1 shows no differences in the quality parameters firmness, titratable acidity (TA), total soluble solids (TSS) and starch index.

3. NOP-1 delayed ripening of tomatoes when applied at the maturity stage ‘breakers’ indicated by slower colour development. Repeated application had no additional delaying effect and did not reach the level of ripening delay induced by 1-MCP. When applied at the maturity stage ‘pink’ no ripening delay was observed. These results indicate that NOP-1 works best when applied at early ripening stages, long before autocatalytic ethylene synthesis takes place.

In general terms, our results show that NOP-1 has promising potential to delay fruit ripening and steer quality of climacteric fruits. Besides significant advances in understanding the technology and the mode-of-action, many details have to be better understood and adapted. In practical terms, more precise information is needed in terms of timing and frequency of application for different species and varieties, and the impact of NOP-1 on the different quality attributes of fruits.

Reifeverzögernde Peptide und ihre Anwendbarkeit zur Steuerung der Qualitätsentwicklung von klimakterischen Früchten

Maßnahmen zur Verlängerung der Haltbarkeit von klimakterischen Früchten spielen eine wichtige Rolle für eine nachhaltigere, globale Lebensmittelversorgung. Ein hoher Anteil der Fruchtverluste ist auf den Reifeprozess und die dadurch bedingten Qualitätsverluste während Transport und Lagerung zurückzuführen. Ethylen ist das Schlüsselhormon für Reifung und Seneszenz. Neuere Studien zum Ethylen-Signalweg haben gezeigt, dass die Signaltransduktion im Wesentlichen von der Interaktion des Ethylenrezeptors (ETR) mit dem Signalprotein EIN2 (Ethylene-insensitive 2) abhängt. Ein neues synthetisches Octapeptid (Nuclear Octapeptid 1, NOP-1), welches vom so genannten Nuclear Localization Signal (NLS) am C-terminalen Ende von EIN2 abgeleitet ist, hemmt die Interaktion zwischen ETR und EIN2 und kann die Fruchtreife verzögern. Hauptziel dieser Studie war die Untersuchung der Reifeverzögerung und der Wirkungsweise des rekombinanten NOP-1 bei Tomaten und Äpfeln. Dosis-Wirkungs- und Zeit-Wirkungs-Experimente wurden durch Aufbringen von NOP-1 in wässriger Lösung auf die Fruchtoberfläche grüner Tomaten durchgeführt. Auf diesen Basisexperimenten aufbauend wurde die NOP-1-Wirkung bei Äpfeln, welche eine deutlich höhere Ethylenproduktion aufweisen als Tomaten, sowie der Einfluss des Peptids auf den Ethylenstoffwechsel, untersucht. Zusätzlich wurden potentielle Effekte unterschiedlicher Applikationsmethoden analysiert. Detaillierte Untersuchungen der NOP-1 Wirkung wurden zudem in verschiedenen Reifestadien von Tomaten und bei wiederholter Applikation durchgeführt. Um eine unmittelbare Kontrolle der Fruchtreifeentwicklung zu ermöglichen wurden sensorgestützte Methoden zur Qualitätsbeurteilung verwendet und diese mit klassischen destruktiven Analyseverfahren verglichen. Zusammenfassend lassen sich die Ergebnisse wie folgt darstellen:

1. NOP-1 in einer Konzentration von 1000 µMol verzögerte die Abreife von Tomaten im Reifestadium ‚mature green' um mehrere Tage, wohingegen Konzentrationen von 400 µM und 2000 µM keinen reifeverzögernden Effekt zeigten. Der Chlorophyllabbau sowie die Akkumulation von Lykopin und ß-Carotin wurden gehemmt, während die Festigkeit nicht beeinträchtigt wurde. Eine Qualitätsminderung der Frucht wurde nicht beobachtet.

2. Bei Äpfeln hemmte NOP-1 (1000 µMol) die Farbentwicklung und den Chlorophyllabbau während der Shelf-life Periode. Dieser Effekt war bei der Oberflächenbehandlung mittels Pinsel (Oberflächenfilm) stärker ausgeprägt als bei Mikrotropfenapplikation (Nachahmung eines Sprühfilms). NOP-1 hatte weder Einfluss auf die Ethylenfreisetzung noch auf die Atmungsaktivität, während 1-MCP beide stark unterdrückte. NOP-1 zeigte keine Unterschiede in den Qualitätsparametern Festigkeit, titrierbarer Säuregehalt (TA), gesamtlösliche Feststoffe (TSS) und Stärkeindex.

3. NOP-1 verzögerte die Abreife bei Tomaten im Reifestadium „Breakers", welches durch die Verzögerung der Fruchtschalenverfärbung dokumentiert wurde. Die wiederholte Applikation hatte keinen zusätzlichen verzögernden Effekt und war geringer als die Reifeverzögerung durch 1-MCP. Im Stadium „Pink" trat keine Reifeverzögerung ein. Dies weist daraufhin, dass die NOP-1 induzierte Reifeverzögerung am stärksten in frühen Reifestadien ist, bevor die autokatalytische Ethylensynthese einsetzt.

Zusammenfassend zeigen die Ergebnisse dieser Studie, dass NOP-1 ein vielversprechendes Potenzial zur Fruchtreifeverzögerung und zur Regulierung der Qualität klimakterischer Früchte hat. Trotz Verbesserung der Kenntnisse über Wirkungsweise und Anwendbarkeit müssen noch viele Details besser verstanden werden. Für die praktische Anwendung sind arten- und sortenspezifische Informationen über Zeitpunkt und Häufigkeit sowie über die NOP-1-Wirkung auf unterschiedliche Qualitätsparameter der Früchte besonders wichtig.

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List of abbreviations

1-MCP	1-methylcyclopropene
a*	red to green
ACC	aminocyclopropane 1-carboxylic acid
ACO	aminocyclopropane 1-carboxylic oxidase
ACS	aminocyclopropane 1- carboxylic synthase
AHP	*Arabidopsis* His Phosphotransfer
ATP	adenosine triphosphate
AtEIN2	*Arabidopsis thaliana EIN 2*
AVG	aminoethoxyvinylglycine
b*	blue to yellow
BGF	blue-green fluorecence
BR	breakers
°C	degree Celcius
CD	circular dichroism spectroscopy
CDD	charged-coupled device
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CO_2	carbon dioxide
CTR1	Constitutive Triple Response
Cu	copper
cv.	cultivar
DAT	day after treatment
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*

EIL	ethylene insensitive 3-like
EIN	ethylene-insensitive protein
ER	endoplasmatic reticulum
ERF	ethylene responsive genes
ERS	ethylene response sensor
et al.	*et alii (m.), et aliae (f.), and others*
ETR	ethylene receptor
FID	flame ionization detector
FLAV	flavonol index, decadic logarithm of the red to ultraviolet excitation ratio of far-red chlorophyll fluorescence
FRET	charged-coupled device
FRF	near-infrared
FRF_UV	near-infrared excited with UV light
FRF_R	near-infrared excited with red light
g	gram
GAF	cyclic guanosine monophosphate phosphodiesterase, adenylyl cyclase, and formate hydrogen lyase transcriptional activator
GC	gas chromatography
h	hours
HCN	hydogen cyanide
i.e.	*id est*, that is
IPTG	isopropyl-ß-d-1-thiogalactopyranosied
kbar	kilo bar
K_d	dissociation constant
kDa	kilodalton
kg	kilogramm
L	liter
L*	lightness

LED	light-emitting diods
LeETR	Tomatoe ethylene receptor
MdETR	Apple ethylene receptor
µMol	micro Mol
O_2	oxygen
µg	microgram
mm	milimeter
mM	mili Mol
mmol L^{-1}	millimoles per liter
mg	milligram
MG	mature green
min	minutes
min.	minimum
mL	millilitre
MW	molecular weight
µL	microliter
nL	nonoliter
mRNA	messenger ribonucleic acid
MST	microscale thermophoresis
N	Newton
NaCL	sodium chloride
Ni-NTA	Nickel-Nitrilotriacetic
NLS	Nuclear localization signal
n	number of replications
nm	nanomete
nM	nano Mol
NO	nitric oxide
NOP-1	synthetic octapeptide

NR	Nuclear receptor
PBS	Phosphate Buffered Saline
PG	Polygalacturonase
pH	*pondus Hydrogenii*
PI	Pink
PMSF	phenylmethylsulfonyl fluoride
%	percent
RAF	rapidly accelerated fibrosarcoma
RF	red fluorescence
RT	room temperature
rpm	rounds per minute
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulfate
SE	standard error
SFR	Simple Fluorescence Ratio
SFR_R	Simple Fluorescence Ratio excited with red light
TA	titratable acidity
Tris/HCL	Tris(Hydroxymethyl)Methylamin-Hydrochlorid
TSS	total soluble solids
UV	ultraviolet
v/v	volume per volume
vs	*versus*
w/v	weight per volume

A Introduction

1. Postharvest losses

Worldwide a great amount of food produced for human consumption are lost or wasted even before the products reach the final consumers. About 50% of those food losses are estimated to be vegetables and fruits. Losses mainly occur during or immediately after harvest, along the supply chain, transport and storage and at the selling points (Blanke 2014). During storage, the physiological mechanisms of maturation and senescence lead to quality decrease and spoilage (Abbott 1999).

Sustained fruit quality is important to avoid food losses (FAO, 2011). The term quality is a complex construct and might refer to nutritional value, sensory attributes like texture, aroma, taste and appearance, and secondary metabolic compounds (Abano and Buah, 2015; Abbott, 1999). Consumers and retailers are mainly focused on high quality standards of fruits and vegetables, which in term leads to wasting of products that do not meet the set standards (Blanke, 2014; FAO, 2011). Ripening delay of climacteric fruits during storage is a common method to maintain fruit quality and extend shelf-life, whereby postharvest losses can be reduced. The impairment or slow-down of ethylene metabolism and cascade signalling by low temperature and controlled atmosphere, or by chemical substances, are common procedures at commercial scales for ripening control (Passam et al., 2007; Saltveit, 2005; Watkins et al., 2000). New insights in the ethylene signalling pathway offers the opportunity for a biological approach of ripening control and quality maintenance of climacteric fruits (Bisson and Groth, 2015).

2. Climacteric fruits

In fruits two different ripening mechanisms can be distinguished (Alexander and Grierson, 2002). Fruits with a strong increase in respiration triggered by an ethylene burst during the ripening phase are defined as climacteric fruits (Alexander and Grierson, 2002) (Fig. 1). Fruits that do not show any significant change in respiration and ethylene levels and stop ripening after harvest are defined as non-climacteric fruits (Alexander and Grierson, 2002) (Fig. 1). The plant hormone ethylene is responsible for the ongoing postharvest maturation process of climacteric fruits (Brady, 1987). The non-climacteric fruits include citrus and strawberry, whereas apple, avocado, banana and tomato belong to the group of climacteric fruits (Blanke, 2014).

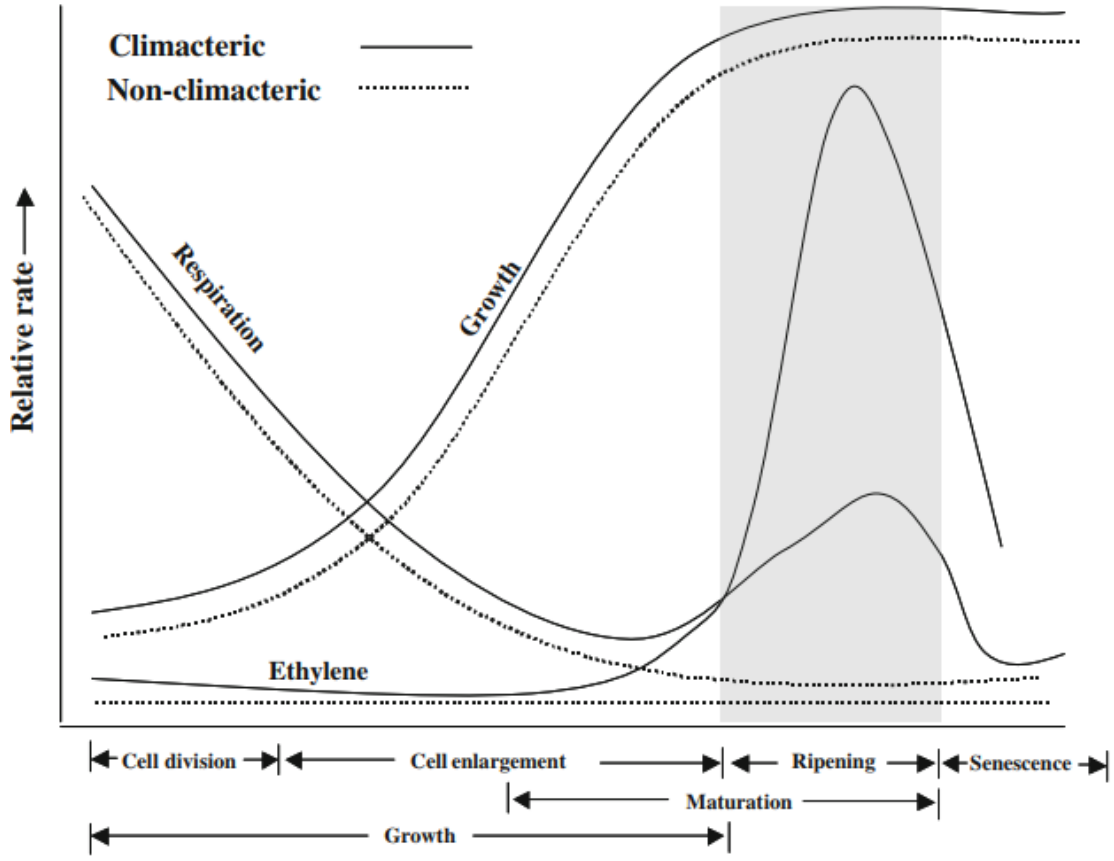

Figure 1. Relative differences between climacteric and non-climacteric fruits from the development until senescence: growth, respiration (CO_2 production or O_2 consumption) and ethylene synthesis (Paul et al. 2012).

2.1 Tomato (*Solanum lycopersicum*): a climacteric fruit with short storage potential

Tomato is one of the most important climacteric fruits and is of high worldwide economic and nutritional importance (Canene-Adams et al., 2005; Vidoz et al., 2010). Tomatoes contain significant amounts of fat- and water- soluble vitamins (A, E, C, folate) and valuable phytochemicals like carotenoids such as lycopene, ß-carotene and pro-vitamin A as well as polyphenols such as flavonols (Canene-Adams et al., 2005). During its ripening, the colour change from green to red makes the tomatoes a suitable model system to study the ripening process as influenced by several pre-harvest and postharvest factors (Alexander and Grierson, 2002; Foolad, 2007). Furthermore, the well-studied genome, which is comparatively small, is well suited for genetic and biochemical studies on the maturation process (Bisson and Groth, 2015; Foolad, 2007).

2.2 Apple (*Malus domestica*): a climacteric fruit with long storage potential

Under the pome fruits, apple is the most consumed fruit and favoured in Germany (Statistisches Bundesamt, 2018). With its high amounts of fibres and phytochemicals acting as antioxidants, apples have a high health benefit for the human diet (Boyer and Liu, 2004). Epidemiological studies indicate a protective value against cardiovascular, and gastrointestinal diseases, and some cancers types (Boyer and Liu, 2004). This is due to high concentration of polyphenols and flavonoids (Boyer and Liu. 2004). The importance of apples for nutrition is reflected in the world production of about 70 million tons per year (USDA, United States Department of Agriculture, 2019). Apple is a good model fruit for detection of ripening and senescence (Yang et al., 2013). Among the climacteric fruits, apples show one of the highest ethylene production during ripening (Blanke, 2014). Among the many varieties of apples, Golden Delicious is grown worldwide and is still one of the most popular cultivars in Europe (Knoche and Khanal, 2011; Xing et al., 2006). It is a frequently used variety for studying ripening behaviour in climacteric fruits (Betemps et al., 2012; Gorny and Kader, 1996; Yuan and Carbaugh, 2007; Zhu et al., 2008).

3. Ripening and quality development

Fruit ripening is characterized by sensoric and structural changes, which occur at the transition from advanced growth stage to senescence and make the fruit attractive to the consumer (Lelievre et al., 1997; Watada et al., 1984). This is a genetically determined development process, which leads to enzyme-induced changes in fruits and vegetables

(Lelievre et al., 1997). This includes alterations in colour, texture, firmness, flavor and aroma (Alexander and Grierson, 2002; Brady, 1987). The physiological, morphological and biochemical changes are consequences of degradation of pectines, cellulose and chlorophyll as well as due to a degradation and consumption of acids and starch, and an increase of sugars, carotenoids and aroma volatiles (Alexander and Grierson, 2002; Brady, 1987). The ripening process is controlled by the so-called ripening- related genes (Abano and Buah, 2015). Activation of hydrolytic enzymes like cellulase and pectinase leads to cell wall degradation and softening (Brady, 1987). Enhanced amylase induces starch degradation with consecutive elevation of sugar content (Medlicott and Thompson, 1985; Xiao et al., 2018). Lowering organic acid content decreases the acidity-sugar ratio, which is an important component of the mature taste (Medlicott and Thompson, 1985). Chlorophyll breakdown is another characteristic of the ripening process. Conversion of chloroplasts to chromoplasts is the consequence (Alexander and Grierson, 2002). In parallel to this conversion, other pigments like lycopenes and ß-carotenes are being synthetized, resulting in the characteristic ripening colour of the fruit (Alexander and Grierson, 2002; Fraser et al., 1994). Raise in the production of aroma volatiles like esters, alcohols, aldehydes, terpenoides and apocarotinoides characterize the odor formation of the ripening fruits (El Hadi et al., 2013). Together with the sugar–acid ratio, they establish the main components of the flavor of the ripe fruit (El Hadi et al., 2013). The ripening process is also accompanied by a collection of so-called valuable compounds like polyphenols and flavonols with antioxidative activity, folate and vitamins (Azeez et al., 2012; Canene-Adams et al., 2005).

Besides decrease in fruit firmness, the ripening of tomatoes is also characterised by colour change. During ripening, the content of chlorophyll decreases, and the content of lycopene and ß-carotene increases – leading to alterations that can be easily detected by the human eye. To differentiate the ripening stages of tomatoes, a colour classification system was established by The United States Department of Agriculture (USDA, 1991). According to this classification system, the tomato fruit ripeness is divided into six maturity stages:

Stage 1 = green, complete mature green fruit

Stage 2 = breakers, colour break from green to red (tannish-yellow, pink). 90% of the surface is still green

Stage 3 = turning, 10% – 30% of surface is redish

Stage 4 = pink, 30% – 60% of surface is redish

Stage 5 = pink, 60% of surface is pinkish red or red

Stage 6 = 100% red surface

In apples, decreases in firmness and starch content as well as change in acid - sugar ratio, are typical events during ripening (Mir et al., 2004; Wu et al., 2007; Yuan and Carbaugh, 2007). The high responsivity of apples to ethylene imply a strong increase in ethylene production and a rise in respiration (Blanke, 2014; Yang et al., 2013).

4. Impact of ethylene on fruit ripening

Essential factors in plant development, such as regulation of maturity and senescence, are controlled by signalling from the plant hormone ethylene (Abano and Buah, 2015; Alexander and Grierson, 2002; Guo and Ecker, 2004). The ripening process is regulated simultaneously by ethylene-dependent and ethylene-independent pathways in both climacteric and non-climacteric fruits (Alexander and Grierson, 2002; Lelievre et al., 1997). Whereas in non-climacteric fruits ethylene production stays very low even during ripening, an ethylene burst is required in climacteric fruits to allow a proper maturation. Ethylene is therefore considered to play a key role as a regulator in terms of genetically programmed fruit ripening processes (Alexander and Grierson, 2002; Pech et al., 2008; Bisson and Groth, 2015).

To understand the complex ripening process, more detailed work on ethylene-induced mechanism is necessary, which in turn is of fundamental relevance for global food production (Alexander and Grierson, 2002; Kessenbrock et al., 2017).

4.1 Ethylene biosynthesis

In general terms, two systems control the ethylene biosynthesis (Bapat et al., 2010; Brady, 1987). The elementary production of the plant hormone ethylene in all tissues of unripe climacteric and non-climacteric fruits is due to system 1, which provides basal ethylene levels and underlies an autoinhibitory regulation (Bapat et al., 2010; Brady, 1987). System 2 is specific to climacteric fruits and refers to ‘autocatalytic synthesis’ of ethylene (Bapat et al., 2010).

In the first step of the ethylene biosynthesis, the aminoacid methionine, which is the initial substance in the pathway of ethylene synthesis, is catalysed via

adenosylmethionine synthetase (SAM synthetase) to S-adenosylmethionine (SAM) (Grierson, 2012). The aminocyclopropane 1-carboxylic synthase (ACS) transforms SAM to aminocyclopropane 1-carboxylic acid (ACC), which is subsequently oxidized by ACO (aminocyclopropane 1-carboxylic oxidase) in an oxygen-dependent reaction to ethylene, CO_2 and HCN (hydrogen cyanide) (Johnson and Ecker, 1998). The step from SAM to ACC is rate limiting, so that the activity of ACS determines the production rate of ethylene (Alexander and Grierson, 2002). Up to nine isoenzymes of ACS and ACO have been identified to date with the largest coding gene family known in tomatoes (Johnson and Ecker, 1998). In plants, the expression of ACC synthase is induced by various environmental factors, such as temperature, dryness, and wounding as well as by developmental and hormonal signals (Johnson and Ecker, 1998; Kadereit et al., 2014) (Fig. 2).

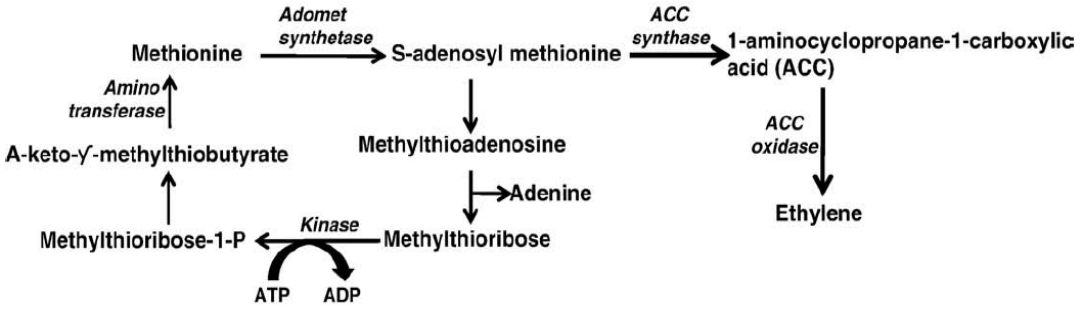

Figure 2. Pathway of ethylene biosynthesis (Bapat et al., 2010).

System 2 of the regulation of ethylene production operates during the ripening phase of climacteric fruits. Here, ACS gene expression is stimulated by ethylene itself causing a positive feedback mechanism (autocatalytic) resulting in the typical ethylene burst (Alexander and Grierson, 2002). The restoration of methionine as precursor for ethylene starts with methylthioadenosine after splitting of ACC from SAM by ACS in the so-called Yang cycle (Fig. 2) (Bapat et al., 2010).

4.2 Ethylene signalling pathway

The ethylene signalling pathway is a complex mechanism which has not been fully understood until today (Alexander and Grierson, 2002; Bisson and Groth, 2015). However, experiments with *Arabidopsis thaliana* mutants identified the signal transduction as a receptor–dependent phosphorylation cascade of regulator proteins (Alexander and Grierson, 2002; Hua et al., 1995). First insights in this process come from

investigations on a special characteristic reaction of seedlings, the so-called “triple response” induced by ethylene. The triple response consists in a decrease of longitudinal growth, hypocotyl swelling and exaggeration of the apical hook (Bisson and Groth, 2015; Guzman and Ecker, 1990; Hua et al., 1995). Experiments on mutants of ethylene receptors and signal proteins revealed a group of proteins necessary for ethylene action (ethylene insensitive, EIN2-EIN7) and a group of proteins showing triple response (constitutive), in functionless state even in the absence of ethylene (Johnson and Ecker, 1998). Such proteins are defined as negative regulators. These are different ethylene receptors and the downstream regulator protein Constitutive Triple Response (CTR1) (Johnson and Ecker, 1998).

The pleiotropic effect of ethylene including the ripening of climacteric fruits is mediated by a family of membrane receptors embedded in the membrane of the endoplasmatic reticulum (ER) (Hwang et al., 2002). In the model plant *Arabidopsis thaliana*, five ethylene receptors (ETR1, ETR2, ERS 1, ERS2 and EIN4) which are widespread in the plant kingdom, have been identified (Johnson and Ecker, 1998; Ju and Chang, 2015). Sequence homology with the different tomato ethylene receptors (LeETR1, LeETR2, NR, LeETR4, LeETR5, LeETR6, and LeETR7) is at least 50%, and the apple ethylene receptor MdETR1 shares about 42 - 84% sequence identity with the corresponding tomato ethylene receptors (LeETR4, NR and LeETR1) (Kessenbrock et al., 2017; Klein et al., 2019a). The receptors consist of a transmembrane and a cytosolic component. The cytosolic component of the receptor functionally represents a two-component- histidine kinase sensor (Hwang et al., 2002; Johnson and Ecker, 1998). The N-terminal transmembrane domain binds ethylene in the presents of copper after homo- or heterodimerisation via disulphide bridges (Ju and Chang, 2015). A GAF-domain (cyclic guanosine monophosphate phosphodiesterase, adenylyl cyclase, and formate hydrogen lyase transcriptional activator) connects the ligand-binding domain with the histidine kinase domain which at the C-terminal end is in turn connected to a receiver domain (response regulator domain) which carries an aspartate residue for the absorption of a phosphate group (Johnson and Ecker, 1998; Ju and Chang, 2015). Whereas ETR1, ETR2 and EIN4 have the receiver domain, ERS1 and ERS2 do not, while ETR2, ERS2 and EIN4 show a degenerated histidine kinase domain.

Because of the structural features, the ethylene receptors are divided into two subfamilies. ETR1 and ERS1 are in subfamily I with sharing all motifs of a functioning histidine kinase, whereas ETR2, ERS2 and EIN4 are in subfamily II in which it is

questionable whether the receptors are involved in the phosphorylation cascades (Johnson and Ecker, 1998; Ju and Chang, 2015). The homo- and heterodimers of the ethylene receptors form clusters and build multi-protein complexes with downstream components of the signal pathway involving Constitutive Triple Response (CTR1), a serin/threonin kinase corresponding to RAF-kinases and Ethylene Insensitive 2 (EIN2), an integral membrane protein (Bisson et al., 2016; Ju and Chang, 2015). Figure 3 schematically shows the sequence and operation of the ethylene signalling pathway (Ju and Chang, 2015).

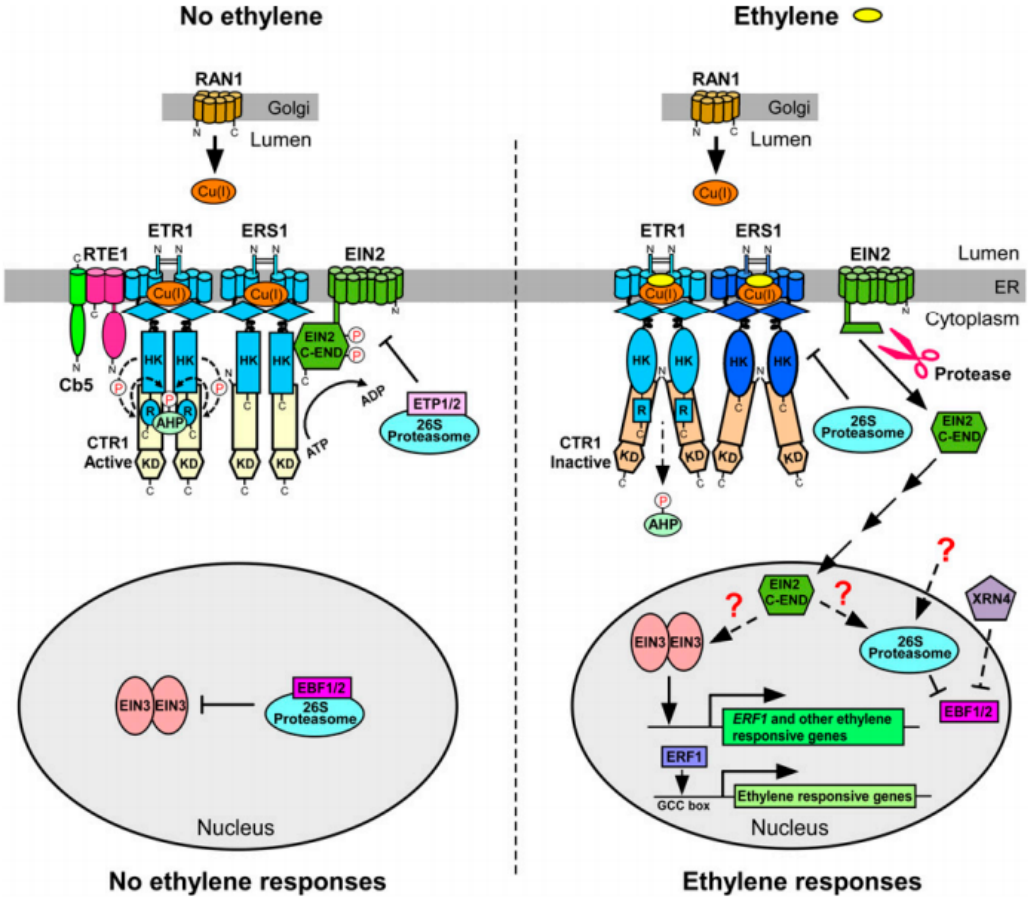

Figure 3. Model of ethylene receptor and ethylene signalling pathway. Copper (Cu) is provided by Responsive to antagonist1 (RAN1) and reversion – to-ethylene sensitivity (RTE1) (Cb5= cytochrome b5). Ethylene receptors (ETR1, ERS1) build homodimers. Left: Absence of ethylene causes autophosphorylation of histidine kinase (HK), phosphorylation of receiver domain (R), *Arabidopsis* HIS phosphotransfer (AHP), CTR1 (KD – kinase domain) and c-terminal end of EIN2. Degradation of EIN2 after targeting by ETP1/2 (EIN2-targeting protein). Right: Presence of ethylene causes dephosphorylisation, cleavage of EIN2 C-END and activation of EIN3 (homodimer). ERF1 (ethylene response factor 1) is build and activates transcription of ethylene response genes. Degradation of transcription factors is inhibited (EIN3 binding F-box = EBF1/2). XRN4 = exoribonuclease for degradation of RNA of EBF1/2 (Ju and Chang, 2015).

The ethylene signalling pathway is negatively regulated. Histidine kinases of the ethylene receptors are active in the absence of ethylene and lead to autophosphorylation with consecutive transfer of the phosphate group to the receiver domain of the receptor or to *Arabidopsis* His Phosphotransfer proteins (AHP) (Johnson and Ecker, 1998; Ju and Chang, 2015). As a result, the CTR1 protein kinase domain is activated which in turn leads to phosphorylation of EIN2 at the C-terminal end on multiple serine and threonine residues (Bisson et al., 2016; Hwang et al., 2002; Ju and Chang, 2015). Phosphorylation of EIN2 inhibits further signal transmission and prepares the protein for proteasomal degradation (Johnson and Ecker, 1998; Ju and Chang, 2015). Binding of ethylene inactivates the receptor which leads to an interruption of the phoshorylation cascade resulting in dephosphorylation of EIN2 (Hwang et al., 2002). Following dephoshorylation, a portion of the C-terminal domain of EIN2 is proteolytically cleaved by a yet unknown protease and then translocated into the nucleus (Bisson et al., 2016; Ju and Chang, 2015). This C-terminal end of 815 amino acids (aa 479 – 1294) contains an octapeptide (H_2N-LKRYKRRL-COOH) called nuclear localisation signal (NLS), which was first discovered in the model plant *Arabidopsis thaliana*. NLS is responsible for translocation of the C-terminal end of EIN2 from the cytosol into the nucleus. Here, interaction with transcription factors ethylene insensitive 3 (EIN3) and ethylene insensitive 3-like (EIL) happens (Bisson and Groth, 2011; Bisson and Groth, 2015). NLS also plays an essential role in complex formation between EIN2 and ETR1 at the ER membrane, as interaction studies have shown (Bisson and Groth, 2015). This is fundamental for the cleavage of C-Terminal end of EIN2 as a central regulator step of ethylene signalling (Bisson and Groth, 2015). Therefore, the NLS-motif of EIN2 is critical in the ethylene signalling pathway in a dual manner, for ETR-EIN2 complex formation and as key element for the transmission of the ethylene signal to the nucleus (Bisson and Groth, 2015).

The C-terminal end of EIN2 activates the transcription factors EIN3/EIL in the nucleus, which in turn induces the transcription of the genes for the ethylene response factors (ERF) (primary responsive genes). The ERF themselves are transcription factors, which activate the ethylene response genes for metabolic and phenotypical changes (Ju and Chang, 2015).

4.3 Ethylene response

The upregulation of ethylene response genes during ripening leads to dramatic changes in many fruit properties (Pech et al., 2008). Chlorophyll degradation, accumulation of carotenoids like lycopene, and ß-carotene and synthesis of aroma volatiles are purely ethylene-dependent parameters as genetic investigations on transgenic plants and mutants of tomatoes, melons and apples have shown (Alexander and Grierson, 2002; Pech et al., 2008). Volatiles are built from unsaturated fatty acids and amino acids through activity of lipoxygenases, deaminases, decarboxylases and alcohol acetyltransferases forming aldehydes, alcohols, and esters (Alexander and Grierson, 2002). The formation of carotenoids is catalysed by ethylene stimulated enzymes like phytoene synthase and lycopene cyclase (Fraser et al., 1994). Chlorophyll degradation is also an enzyme-linked, ethylene-controlled process, which starts with the activation of chlorophyllase in the chloroplast (Alexander and Grierson, 2002).

However, there is also an ethylene-independent regulation of climacteric fruit ripening, where the initiation of the climacteric process, the loss of acidity and the accumulation of sugars do not require ethylene (Pech et al., 2008). Furthermore, the process of fruit softening has ethylene independent and dependent components. The cell wall degradation is caused by a number of enzymes, like polygalacturonases, xyloglucan endotransglycosylase/ hydrolases, expansin and b-galactosidases (Pech et al., 2008). It has been suggested, that each family of cell wall related genes contains ethylene-dependent, ethylene-independent or partially ethylene-dependent genes (Nishiyama et al., 2007; Pech et al., 2008). However, the gene that encodes for polygalacturonase (PG), the major enzyme for pectin degradation, is characterized as ethylene -dependent. Studies on transgenic ACC tomatoes have shown that the accumulation of PG mRNA is ethylene-dependent (Sitrit and Bennett, 1998). However, although ethylene is the dominant trigger for ripening in climacteric fruits, it has been suggested that both ethylene-dependent and ethylene-independent gene regulation pathways coexist to co-ordinate the process in climacteric and non-climacteric fruits (Alexander and Grierson, 2002; Lelievre et al., 1997).

5. Control of fruit ripening

Control of ripening is of high importance to ensure fruit quality and to decrease post-harvest losses of climacteric fruits. With the knowledge on the role of ethylene in fruit ripening, methods that limit ethylene synthesis and ethylene responses are actively adopted. Storage of fruits at low temperatures under controlled atmosphere is a common procedure at commercial scales to steer the ripening process. Low temperatures reduce respiration rates and ethylene production (Gorny and Kader, 1996; Yahia et al., 2004). Furthermore, low levels of O_2 and elevated concentration of CO_2 inhibit the conversion from ACC to ethylene (Gorny and Kader, 1996; Passam et al., 2007).

Genetic engineering as an alternative approach for ripening control is increasingly used with ongoing developments, despite ongoing critical discussions in Europe about the general handling with genetically engineered food. Retardation of ethylene biosynthesis by suppression of gene expression of key enzymes for ethylene formation, or insertion of genes that encode for enzymes which degrade ethylene precursors like ACC deaminase or SAM hydrolase, are meanwhile established methods (Pathak et al., 2018). Control of ethylene perception by genetically linked ethylene receptor modification and suppression of polygalacturonase activity are also tools for ripening delay (Pathak et al., 2018).

Chemical methods to manage the ripening process of climacteric fruits consist of the application of ethylene synthesis inhibitors like aminoethoxyvinylglycine (AVG) or nitric oxide (NO), and the application of substances which inhibit the ethylene perception like 1-methylcyclopropene (1-MCP). At present, these two ethylene inhibitors are mainly used for commercial practice (Saltveit, 2005; Watkins et al., 2000; Yuan and Carbaugh, 2007). In addition, silver salts (silver thiosulfate, silver nitrate; Ag) also block ethylene receptors. But these chemical inhibitors are partly toxic and difficult to apply due to their gaseous or hydrophobic character (Saltveit, 2005; Watkins et al., 2000; Yuan and Carbaugh, 2007). This limits their field of application and makes their use technically complex.

5.1 1-methylcyclopropene (1-MCP)

1-methylcyclopropene (1-MCP) is commercially used for extending shelf-life and ensuring the quality of several climacteric fruits (Blankenship and Dole, 2003). In Germany, it is authorized for use on apples and pears; in other countries it is also used

on kiwis, plums, avocados, bananas, kakis, strawberries and tomatoes (Watkins et al., 2000; Blankenship and Dole, 2003). 1-MCP is a small unsaturated, cyclic hydrocarbon with a molecular formula of C_4H_6 in a gaseous aggregate state (Blankenship and Dole, 2003). It acts as an antagonist at the ethylene receptors because of its chemical similarity to ethylene (C_2H_4) (Blankenship and Dole, 2003). The affinity of 1-MCP to the ethylene receptors is 10 times greater than of ethylene (Blankenship and Dole, 2003). Binding is irreversible and recovery to ethylene sensitivity depends on the production of new receptors (Blankenship and Dole, 2003; Watkins et al., 2000). Therefore, the duration of 1-MCP action is specific to fruit varieties (Blankenship and Dole, 2003). Avocados show a two-week maturity delayed effect, whereas ripening delay on tomatoes is about 5-10 days (Blankenship and Dole, 2003). Repeated applications with 1-MCP leads to an extension of the effect (Mir et al., 2004).

For commercial use, 1-MCP is included in cyclic oligosaccharides in powder form (Watkins et al., 2000). By adding water, 1-MCP is released as a gas and diffuses easily through the fruit peel (Blankenship and Dole, 2003). Exposure time of fruits should be between 12 – 24 h. 1-MCP evaporates quickly and is safe for humans, animals and the environment (Blankenship and Dole, 2003). In some fruit varieties, 1-MCP has shown negative effects related to increased rate of diseases and disorders (Blankenship and Dole, 2003). Examples on that include increase of decay in strawberry, higher rate of brown core in apples and higher susceptibility to *Botrytis cinerea* in tomatoes (Blankenship and Dole, 2003; Höhn et al., 2007; Diaz et al., 2002).

5.2 Aminoethoxyvinylglycine (AVG)

AVG is a small water-soluble molecule that inhibits ACC-synthase, the key-enzyme of the ethylene biosynthesis (Blanke, 2014; Zhu et al., 2008). It is mainly used in pome fruits like apples and pears in the pre-harvest setting for reduction of fruit drop before harvest (Blanke, 2014; Byers, 1997). Nevertheless, AVG has shown on plums, that application of AVG before harvest has a benefit on fruit shelf-life. The treated fruits showed a delaying of ripening during storage, especially the fruit firmness was affected (Jobling et al., 2003). Reduction of ethylene synthesis is about 60-90% depending on cultivar and AVG concentration (Saltveit, 2005). When applied four weeks before harvest, a maturation delay of 7-10 days can be achieved (Yuan and Carbaugh, 2007). However, the influence of ACC leads to the inhibition of ethylene synthesis, whereas the perception of external ethylene is not impaired (Klee et al., 1991).

6. Novel NLS derived octapeptide for ripening inhibition

A new way of influencing maturity of fruits and vegetables has been discovered based on a previously unknown function of the NLS-motif in EIN2 (Bisson and Groth, 2011). After detection of the sequence of the NLS-motif of the C-terminal part of EIN2 (aa 1261 – 1268), localisation studies with fluorescence tagged C-terminal end of EIN2 containing or lacking NLS-motif show that NLS is essential not only for the nuclear transport, but also for the interaction between EIN2 and ETR1 (Bisson and Groth, 2015). It has been shown that peptides which mimic the NLS-motif have the property to influence the interaction between ETR1 and EIN2. The nuclear localisation signal octapeptide 1 (NOP-1) is such a peptide which interferes with the protein-protein interaction leading to impairment of ethylene signalling with consecutive inhibition of ripening (Bisson et al., 2016; Kessenbrock et al., 2017). Thereby, the inhibitory effect of NOP-1 acts downstream of CTR-1 (Bisson and Groth, 2015; Bisson et al., 2016).

With the information on the role of NLS in the ethylene signalling pathway, the synthetic peptide NOP-1 has been engineered for inhibition of the ETR1-EIN2 interaction. NOP-1 is an analogue peptide of the NLS-motif found in *Arabidopsis thaliana*. The NOP-1 has the same amino acid sequence as the NLS-motif (LKRYKRRL) (Bisson and Groth, 2015). Several other synthetic octapeptides, all mimicking the NLS-motif and differing only in one to two amino acids at different positions, showed inhibitory effects regarding EIN2 – ETR1 interaction (Bisson and Groth, 2015). Results suggest that the charge of the peptide is crucial for the binding force on ETR1, being more important than the exact sequence.

The NLS-motif consists predominantly (62%) of basic amino acids that confer, a positive charge (Bisson and Groth, 2015). In contrast, negatively charged octapeptides showed no inhibitory effect on the ETR1 – EIN2 interaction (Bisson and Groth, 2015). The inhibitory mechanism of NOP-1 is competitive through blocking the binding site of the C-terminal end of EIN2 at the GAF-domain of ETR1 (Bisson and Groth, 2015) (Fig. 4).

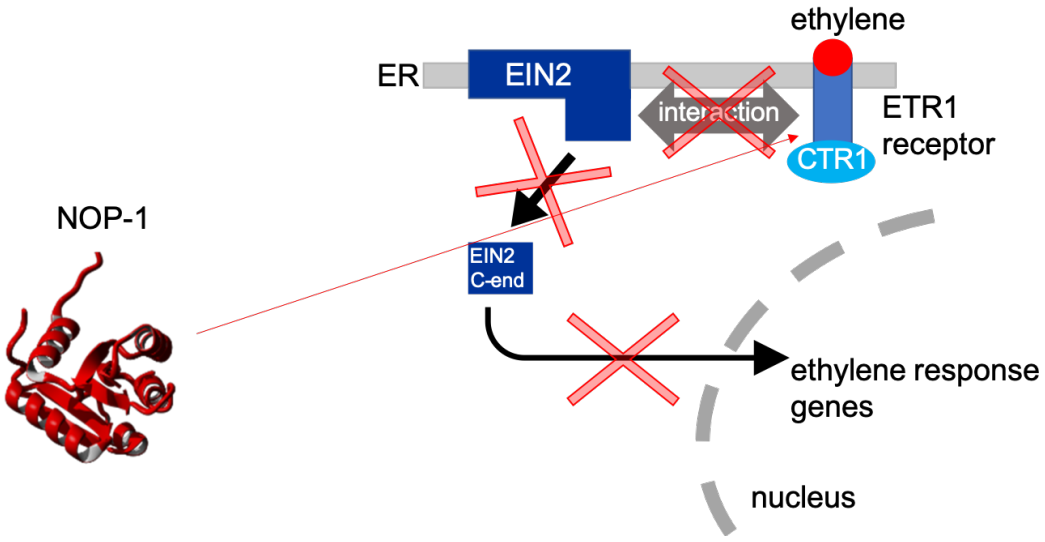

Figure 4. Model of target point of NOP-1 in the ethylene signalling pathway. NOP-1 attaches to ETR1 and blocks the interaction between ETR1 and EIN2 (ethylene insensitive). NOP-1 inhibits downstream of CTR1 (Constitutive Triple Response) the ethylene signalling pathway (adapted by Bisson and Groth, 2010; Bisson and Groth, 2015).

Because the NLS-motif of EIN2 is highly conserved in the plant kingdom, the inhibitory ripening effect of NOP-1 which is derived from the NLS-motif in *Arabidopsis thaliana* is transferable to other species allowing post-harvest experiments e.g., tomatoes and apples (Bisson et al., 2016; Kessenbrock et al., 2017; Klein et al., 2019a). These experiments demonstrate that NOP-1 delays the ripening process of climacteric fruits (Bisson et al., 2016; Kessenbrock et al., 2017; Klein et al., 2019a). The application of NOP-1 on tomatoes and apples takes place in an aqueous solution in which the crystalline NOP-1 is dissolved (Kessenbrock et al., 2017; Klein et al., 2019a). Based on the experimental results it is assumed that the NOP-1 peptide permeates the cuticle and is taken into the cells, possibly by oligopeptide transporters, which are responsible for peptide transport (Bisson et al., 2016; Klein et al., 2019a). A direct uptake on the basis of the inherent molecular properties (short; water solubility; partly hydrophobic and/or polybasic structure; positive net charge of physiological pH) is also possible, but the exact mechanism is still unclear (Bisson et al., 2016; Järver and Langel, 2006). Because of the high solubility of NOP-1 in water, it might be possible to use NOP-1 as sprayable solution on pre-harvest to optimize the harvest management and fruit quality on the trees. For controlling the ripening and quality in postharvest and shelf-life, the NOP-1 solution allows the application as spray and immersion bath (Bisson et al., 2016; Kessenbrock et al., 2017; Klein et al., 2019a; Klein et al., 2019b).

NOP-1 and 1-MCP inhibit different target points in the ethylene pathway. 1-MCP blocks the ethylene receptor, so that ethylene cannot start the ethylene signalling pathway

(Blankenship and Dole, 2003). This mechanism also reduces ethylene production (Blankenship and Dole, 2003). In contrast, NOP-1 causes downstream impairment through an inhibition of ETR1–EIN2 interaction, which thereby causes a disturbance of the ethylene signalling pathway (Bisson and Groth, 2015; Klein et al., 2019a). In comparison to 1-MCP, the autocatalytic ethylene production will not be affected by NOP-1 (Bisson et al., 2016).

7. Detection of changes in maturity and quality parameters

A proper way to assess the effect of ripening inhibiting substances like NOP-1, is to track the ripening development of fruits over the time. Sensory properties can be perceived by humans, whereas nutritional values cannot be directly grasped by the human senses (Shewfelt, 1999). Moreover, human judgement usually does not provide an objective assessment. Therefore, objective methods have to be selected to ensure comparable evaluations. Besides the traditional destructive measurements of firmness, content of sugars, acids, vitamins and pigments, the changes in fruit ripening might be also evaluated over the time by non-destructive techniques (Abbott, 1999; Hoffmann et al., 2015). The adoption of both destructive and non-destructive techniques, simultaneously, provides a range of parameters to assess fruit quality (Abbott, 1999; Hoffmann et al., 2015).

7.1 Sensor based non-destructive determinations

Optical sensors based on reflection or fluorescence determination can be used for a fast and reliable non-destructive determination of fruit ripening and quality (Ben Ghozlen et al., 2010; Cerovic et al., 2008; Hoffmann et al., 2015; McGuire, 1992). The monitoring parameters of the optical sensors strongly correlate with the analytical values (Hoffmann et al., 2015; McGuire, 1992). Skin colour is one of the most important indicators of fruit ripening (Fraser et al., 1994; Hoffmann et al., 2015) while the colour change during ripening is caused by chlorophyll degradation and lycopene and ß-carotene accumulation (Abbott, 1999). A portable spectrophotometer (CM-503d, Konica Minolta Inc., Tokyo, Japan), whose principle is based on detecting the light reflection of colour, can be used for such monitoring (Hoffmann et al., 2015; McGuire, 1992). In addition, the measured parameters can be used for optical ripening and quality evaluation of fruits such as apples and tomatoes (Arias et al., 2000; Funke and Blanke, 2005; Hoffmann et al., 2015; López Camelo and Gómez, 2004). Using the CIEL*a*b colour-model (CIE= International Committee for Lighting and Coloring), the measured reflection values can be displayed

in a three-dimensional space. Thereby, the parameter L for brightness, and the parameters a * and b * which indicate the position of the colour coordinates, are recorded (McGuire, 1992). Skin colour change can be determined by the hue index, which sets the tone as an angle measure (Hoffmann et al., 2015). The parameter a* is the point of the green-red axis and the parameter b* is defined as the point of the yellow-blue axis. Thereby, the hue angle is calculated from the arc tangent of the b*/a* ratio (tan-1($b*a*$)) where, for example, 0° for red-purple, 90° for yellow, 180° for green-blue and 270° for blue is represented (McGuire, 1992). López Camelo and Gómez (2004) showed that the hue index can measure the colour changes of tomatoes during the ripening process.

Beside the measurement of light reflection, fluorescence detection also allows the estimation of fruit ripening and quality (Ben Ghozlen et al., 2010; Cerovic et al., 2008). Using optical multispectral sensors, fluorescence in different wavelengths can be recorded (Ben Ghozlen et al., 2010). The fluorometric sensor screens the chlorophyll fluorescence and estimates the chlorophyll and flavonol as well as the anthocyanin content by stimulation of the pigments with different excitation lights (UV, red, green, blue) (Cerovic et al., 2008; Hoffmann et al., 2015). The fluorescence is detected in three spectral regions; blue-green (425–475 nm), red (680-690 nm) and far-red (720-755 nm) (Ben Ghozlen et al., 2010). The index SFR (Simple Fluorescence Ratio) is determined by the ratio of near-infrared (FRF) to red fluorescence (RF) emission after excitation by red light and estimates the chlorophyll content. Thereby, as higher the fluorescence ratio FRF/RF, as higher the chlorophyll content, due to the reabsorption of emitted red fluorescence light by chlorophyll (Ben Ghozlen et al., 2010). Based on the shielding function of polyphenols, estimations of the epidermal flavonol and anthocyanin content are possible. Determination of the epidermal pigment content of flavonoids and anthocyanins is achieved by comparing the near-infrared fluorescence of chlorophyll after excitation with two different wavelengths. Flavonols absorb light in the UV-A spectral area. While red light reaches the chlorophyll, UV light is absorbed by the upper-localized flavonols. Based on the recorded fluorescence intensities, the corresponding indices for the estimation of epidermal flavonol content according to Lambert's Beer's law from the decadic logarithm of the quotient of near-infrared fluorescence (FRF = far red fluorescence) excited with red, and the near-infrared fluorescence excited with UV-A light, are calculated (FRF_UV) (flavonol = log (FRF_R/FRF_UV) (Ben Ghozlen et al., 2010; Betemps et al., 2012).

7.2 Ethylene detection by gas chromatography

In addition to the measurement of the changes in the pigments, in sugar-acid ratio or in firmness, the detection of the ripening gas ethylene leads to very reliable statements about the maturation phase of the fruit (Janssen et al., 2014; Makky et al., 2014; Paul et al., 2012; Saquet, 2016; Tatsuki et al., 2007; Yang et al., 2013). Ethylene is typically quantified by gas chromatography (Grierson, 2012; Janssen et al., 2014; Yang et al., 2013; Makky et al., 2014). Gas chromatography examines volatile mixtures for their individual components and allows qualitative and quantitative analysis of volatile substances (Cristescu et al., 2013; Ehlers, 2008; Janssen et al., 2014). With a carrier gas (mobile phase), which is usually helium or nitrogen due to their inert property, the gas mixture is sent through a heated column. The column is coated with an adsorbent substance (stationary phase) which separates the gas mixture due to the different molecule's properties of different substances. Due to the varying absorption behaviour on the stationary phase the components flow at different rates through the column, which is referred to as the retention time. At the end of the column, the detector measures the intensity and time of the individual components.

Based on the retention times, each peak in the output signal can be assigned to a specific gas component. The intensity at which the output signal appears, indicates the concentration of the sample (Ehlers, 2008; Janssen et al., 2014). The capillary column and the packed column are two kinds of columns which are available for GC analysis (Janssen et al., 2014). The packed columns are very robust and usually made of glass or metal with a length of 1-3 meters. They are filled with a solid support material such as activated alumina or with a solid support such as diatomaceous earth plus a liquid film. Capillary columns, on the other hand, are many times thinner and longer, and the stationary phase consists of a thin liquid film, mostly of polyethylene glycols (Ehlers, 2008; Janssen et al., 2014). A variety of detectors can be combined with the GC, but in general, the FID (flame ionization detector) is most suitable for detecting ethylene (Cristescu et al., 2013). The evaluation of the chromatograms can be carried out quantitatively on the peak area comparison of the sample and an internal standard (Ehlers, 2008).

In addition to the determination of ethylene, the measurement of respiration, which is measured by an infrared gas analyser, also provides information about the ripening stage of the fruit (Klein et al., 2019a).

8. Objectives of this study

Inhibition of ethylene signalling through impairment of protein-protein-interaction between ethylene receptor (ETR) and ethylene insensitive 2 (EIN2) due to oligopeptides derived from the NLS-motif of EIN2 is a new approach for ripening control of climacteric fruits. The recombinant octapeptide NOP-1 mimicking the conserved NLS-motif of EIN2 in *Arabidopsis thaliana* showed prevention of EIN2 – ETR-1 complex building in biochemical studies on tomatoes (Bisson et al., 2016).

The aim of the present work was to evaluate this novel technology for ethylene signalling inhibition in order to steer the fruit quality under practical conditions. In this study, the mode - of - action of NOP-1 on tomato and apple physiology was investigated.

Different concentrations of aqueous peptide solutions and different application techniques were used to create dose response curves and time response curves to estimate application optima and duration of the effects.

Bio-efficacy of the peptide was evaluated on basis of physiological and biochemical parameters. Degradation of chlorophyll, synthesis of carotenoids, fruit firmness and content of sugar and acids was determined. Non-invasive sensor techniques based on light reflection and fluorescence were used to monitor skin colour and chlorophyll content change to allow continuous access to the maturity development. Ethylene release and respiration activity were monitored to evaluate the influence of the peptide on the ethylene signal cascade. Thereby non-treated fruits and fruits treated with 1-MCP were used as reference. In addition to ripening delay, classic consumer-relevant quality attributes were evaluated and compared.

In summary, the peptide-based impairment of ethylene signalling represents a conceptually novel possibility to control plant ethylene response that does not rely on using chemicals or transgenic techniques for preventing spoilage of fruits and vegetables. The main objective of this research was a proof-of-concept of the new biological ripening control method; focus of the single chapters were as follows:

1. NOP-1 surface application on postharvest tomatoes leads to a significant ripening delay. The effectiveness of the delay depends on the NOP-1 dosage without impairing the fruit quality of the fully ripe tomato.

2. NOP-1 treatment of apples before long-term cold storage extends shelf-life and

does not impair the ethylene metabolism. Application technology influences the effectiveness.

3. NOP-1 application at different maturity stages and repeated times, influences the ripening of tomatoes without affecting quality attributes.

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B Novel protein-protein inhibitor based approach to control plant ethylene responses: synthetic peptides for ripening control[1]

1. Introduction

Worldwide, a tremendous amount of food produced for human consumption is lost or wasted until the product reaches the consumer, with about 50% of those food losses being valuable vegetables and fruits (Blanke, 2014). The tomato fruit is one of the most important climacteric fruits (Jackman et al., 1990; Vidoz et al., 2010) and has worldwide high economic and nutritional importance, mainly because of its high concentrations of carotenoids such as lycopene, ß-carotene and pro-vitamin A (Canene-Adams et al., 2005; Vidoz et al., 2010) which accumulate during fruit ripening. The ripening process of climacteric fruits is characterized by a strong increase in cell respiration which is mainly regulated by the plant hormone ethylene (Alexander and Grierson, 2002; Guo and Ecker, 2004). Ripening is initiated by a burst of an auto-stimulated ethylene synthesis, with following activation of ripening related genes (Abano and Buah, 2015). This ethylene related gene expression leads to physiological, morphological and biochemical changes. In the process of fruit ripening, fruits change color, texture, firmness, flavor and aroma (Brady, 1987; Alexander and Grierson, 2002) due to degradation of pectins, cellulose and chlorophyll as well as due to a decreasing content of organic acids and increasing concentration of soluble sugars, carotenes and aroma volatiles (Brady, 1987).

Besides the traditional quality analysis of firmness and content of sugars, acids, vitamins and pigments, changes in fruit ripening and fruit quality might be evaluated by non-destructive optical methods (Abbott, 1999; Hoffmann et al., 2015). Analogous to that, consumers usually estimate fruit quality based on fruit skin color. Color development of tomatoes from green to red can be measured by monitoring chlorophyll degradation as well as lycopene and ß-carotene accumulation (Alexander and Grierson, 2002). Typically, the total content of these pigments is analyzed using wet chemical procedures (Barros et al., 2007; Azeez et al., 2012; Kalogeropoulos et al., 2012), whereas non-

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destructive optical sensors that evaluate overall changes in fruit color based on reflection and fluorescence properties provide monitoring parameters that strongly correlate with the analytical values (McGuire, 1992; Hoffmann et al., 2015).

Control of ripening is important to ensure quality and to reduce post-harvest losses of climacteric fruits. At commercial scales, fruits are usually stored at low temperatures under controlled atmosphere to limit ethylene production and ethylene response (Watkins et al., 2000; Saltveit, 2005; Passam et al., 2007). Alternative approaches, such as genetic engineering of ethylene biosynthesis to decrease endogenous ethylene production, are under development in science and research despite of ongoing discussion in Europe about genetic engineering in general. At the production scale, fruit maturation can be delayed with aminoethoxyvinylglycine (AVG), an inhibitor of ACC-synthase, the key enzyme of ethylene biosynthesis (Saltveit, 2005). For postharvest treatment, 1-methylcyclopropene (1-MCP), a gaseous chemical with the ability to inhibit ethylene receptors and receptor-triggered ethylene response can be applied (Watkins et al., 2000; Yuan and Carbaugh, 2007).

Pathways and mechanisms for biosynthesis, perception and signal transduction of the plant hormone ethylene have been extensively studied in the model plant *Arabidopsis thaliana*. These studies disclosed that the ethylene signal is perceived by a family of five receptor proteins, which form homo- and heterodimers at the ER membrane and function as negative regulators of the ethylene response (Bleecker et al., 1988; Chang et al., 1993; Hua et al., 1995, 1998; Hua and Meyerowitz, 1998; Grefen et al., 2008). Although the exact output of the receptors is still obscure, genetic studies demonstrate that in the absence of ethylene, receptors activate the Raf-like protein kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), another negative regulator of the pathway (Kieber et al., 1993). Downstream of the receptors and the ER associated CTR1 kinase the membrane protein ETHYLENE INSENSITIVE 2 (EIN2), which contains a highly conserved NLS (Bisson and Groth,2011; Qiao et al., 2012) shown to mediate interaction with the up-stream receptors (Bisson and Groth, 2015; Bisson et al., 2016), implements a positive regulatory role on ethylene signalling. In the presence of ethylene, the receptors bind the hormone and become inactivated. CTR1 cannot be activated by the receptors, and the lack of CTR1 activation cannot phosphorylate EIN2. Subsequently, the C-terminal end of EIN2 (C-END) containing the NLS-motif is cleaved off by an unknown mechanism and translocated to the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). In the nucleus, the EIN2 C-terminus directly or indirectly stabilizes the transcription factor

EIN3 (Wen et al., 2012; Li et al., 2015) and its paralogous, the EIN3-like proteins (EILs), and transcription of ethylene response genes is activated (Chao et al., 1997; Solano et al., 1998).

In analogy to the model plant *Arabidopsis*, tomato contains a multigene family of the ethylene receptors. In total, seven isoforms named LeETR1, LeETR2, NR, LeETR4, LeETR5, LeETR6, and LeETR7 have been identified (Wilkinson et al., 1995; Zhou et al., 1996 a, b; Lashbrook et al., 1998; Tieman and Klee, 1999) which are structurally diverse sharing at the most extreme less than 50% sequence identity. Similar to their *Arabidopsis* relatives the tomato receptors cluster in two subfamilies. LeETR1, LeETR2 and NR forming the subfamily I are characterized by a functional histidine kinase domain and a sensor domain consisting of three transmembrane helices. An additional putative membrane-spanning domain is present in LeETR5, LeETR6, LeETR7, and possibly in LeETR4 of subfamily II which are further characterized by a degenerated histidine kinase domain. All receptors except for NR contain a C-terminal response regulator domain (Lashbrook et al., 1998; Tieman and Klee, 1999). Expression patterns vary among the different receptor isoforms. While LeETR1 is expressed constitutively in all tissues, expression of LeETR2 is bound to seed germination and leaf senescence. NR, LeETR4 and to a lower extent LeETR5 are found at high expression levels in ripening fruit (Payton et al., 1996; Lashbrook et al., 1998; Tieman and Klee, 1999), but are rapidly degraded in the presence of ethylene by a 26S proteasome dependent pathway (Kevany et al., 2007). Due to this strong post-translational regulation of their protein level by the plant hormone and the observed correlation of receptor content and fruit ripening (Kevany et al., 2007), these receptors are of particular interest for studying the molecular effect of ripening inhibitors targeting ethylene signalling.

Recent insights in the ethylene signalling pathway propose a novel way to interfere with fruit ripening based on a yet unknown function of the NLS in the ethylene signalling protein EIN2. Peptides such as the synthetic octapeptide LKRYKRRL (NOP-1) mimicking this NLS motif were shown to block the interaction of EIN2 and ETR1 receptors and reduce plant ethylene responses (Bisson and Groth, 2015; Bisson et al., 2016).

In this study, we demonstrate that the NOP-1 octapeptide also efficiently binds to the ripening related tomato receptors NR and LeETR4 structurally divergent from ETR1. Moreover, we provide quantitative measures of the ripening delay related to NOP-1 treatment such as pigment content, overall color analysis and fruit firmness. Our data

show that surface application of NOP-1 on tomato fruits can delay ripening without impairment of fruit quality.

2. Material and methods

Cloning of tomato receptors LeETR4 and NR into expression vector pET16b

Full-length codon optimized cDNA sequences encoding tomato ethylene receptors LeETR4 and NR (UniProt ID: LeETR4 Q9XET8; NR Q41341) were ordered at GenScript United States according to published sequences (NCBI ID: LeETR4 NM_001247276.2; NR NM_001246965.2). Construction of expression vector pET16b (Novagen, Madison, WI, United States) carrying the target DNA sequence, an ampicillin resistance and a deca-histidine tag were performed by Gibson Assembly (Gibson et al., 2009). For amplification of linearized vector forward primer 5'-GGATCCGGCTGCTAACAAAGC-3' and reverse primer 5'-ATGACGACCTTCGATATGGC-3' were used. LeETR4 was amplified using forward primer5'-ATCGAAGGTCGTCATATGCTGCGTACCCTGGCGAG-3' and reverse primer sequence 5'-TTAGCAGCCGCCTTACATCAGAGCTGGATTACGGCTACCACGCA-3'. For amplification of NR forward primer 5'-ATCGAAGGTCGTCATATGGACGATTGCATT-3' and reverse primer 5'-TTAGCAGCCGGATCCTTACAGGCTACGCTGATAACGCT-3' were used. Amplified fragments were added to Gibson Assembly Master Mix containing an exonuclease, a DNA polymerase and a ligase to assemble a circular plasmid with LeETR4 and NR coding sequence, respectively. Reaction assays were incubated at 50°C for 10 min and at 40°C for 60 min. Assembled plasmids were transformed into *E. coli* strain XL 1-blue and sequenced by Seqlab (Göttingen, Germany) to verify correctness.

Expression of recombinant tomato receptors LeETR4 and NR in *E. coli*

For expression of recombinant LeETR4 and NR the related pET16b expression vectors were transformed into *E. coli* strains C43 and BL21 (DE3), respectively. Cells were grown in 2YT medium (1.6% (w/v) peptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCl) with 2% ethanol and 100 µg/mL ampicillin at 30°C. At OD600 = 0.4 temperature was reduced to 16°C. Expression of tomato receptors was induced at OD600 = 0.6 by the addition of 0.5 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG). Cells were grown and harvested after 20 h (LeETR4) or 6 h (NR) by centrifugation for 15 min at 7,000 ×

g and 4°C. Expression of tomato receptors was analysed by SDS-PAGE (Laemmli, 1970) and detected by Western blotting (Towbin et al., 1979).

Solubilization and purification of recombinant tomato receptors LeETR4 and NR

The resulting cell pellet after expression was resuspended in PBS pH 8, 10% (w/v) glycerol, 1 mM dithiothreitol and 0.002% (w/v) phenylmethylsulfonyl fluoride (PMSF). DNase I (10 µg/mL) was added before cells were broken with Constants Cell Disruption System (Constant Systems, Daventry) at 2.4 kbar and 5 °C. Cell Lysate was centrifuged for 30 min at 14,000 × g and 4°C. The resulting supernatant was centrifuged again for 30 min at 40,000 × g and 4°C. The pellet was resuspended in PBS buffer and centrifuged for 30 min at 34,000 × g and 4°C. For solubilisation the pellet was resuspended in 50 mM Tris/HCl pH 8, 200 mM NaCl, 1.2% (w/v) FosCholine-16, 0.002% (w/v) PMSF (buffer S) and stirred at RT and 700 rpm for 1 h. Membrane fragments were isolated by ultracentrifugation (229,600 × g, 4°C, 30 min). The supernatant was loaded to a 5 mL HisTrap FF column operated by an ÄKTAprime plus (both GE Healthcare Life Sciences) at 4°C equilibrated with buffer A (buffer S containing 0.015% (w/v) FosCholine-16), followed by an ATP washing step of 20 column volumes (50 mM ATP, 200 mM NaCl, 50 mM KCl, 20 mM MgCl2, 10 mM ATP and 0.002% (w/v) PMSF). The column was washed with 50 mM imidazole and receptors eluted with 250 mM imidazole. Purified proteins were concentrated to 2.5 mL and buffer was changed to 100 mM potassium phosphate buffer pH 7.3, 300 mM NaCl, 0.015% (w/v) FosCholine-16, 0.002% (w/v) PMSF for labeling with Alexa Fluor 488-Maleimide (Thermo Fisher Scientific) on a PD-10 column (GE Healthcare Life Sciences). Alexa Fluor 488-Maleimide was applied to the protein in 2.5-fold excess and incubated for 30 min at RT. Then, buffer was changed to 50 mM Tris/HCl pH 8, 300 mM NaCl, 5% (w/v) glycerol, 0,015% (w/v) FosCholine-16, 0,002% (w/v) PMSF. Purity of LeETR4 and NR was analyzed by SDS-PAGE (Laemmli, 1970) with colloidal Coomassie staining (Dyballa and Metzger, 2009) and Western blotting (Towbin et al., 1979) using a directly conjugated Anti-His-HRP monoclonal antibody (Miltenyi Biotech, Bergisch Gladbach, Germany). Proper folding of receptors was verified by CD-spectroscopy (Classen and Groth, 2012; Kessenbrock and Groth, 2017).

CD spectroscopy of recombinant tomato receptors

CD measurements were performed in a Jasco J715 spectropolarimeter (Jasco GmbH, Gross-Umstadt, Germany). For the far UV spectra, a cylindrical quartz cuvette from

Hellma Analytics (Muellheim, Germany) with 1-mm-path-length was used. Purified tomato receptors LeETR4 and NR were dissolved to a final concentration of 0.2 mg ml-1 in 10 mM potassium phosphate pH 8.0 and 0.0075% (w/v) FosCholine-16. Protein and FosCholine-16 concentrations were determined by a Direct Detect Infrared Spectrometer (Merck Chemicals GmbH, Darmstadt, Germany) (Strug et al., 2014). For detailed information on protein preparation see Kessenbrock and Groth (2017). Measurements were run at ambient temperature. Each protein sample was recorded in the range of 260 – 185 nm. The CD spectra were obtained by averaging ten individual spectra using a bandwidth of 1 nm at 50 nm min^{-1}. Secondary structure content of purified proteins were calculated from the spectra by CDSSTR and CONTINLL (Provencher and Gloeckner, 1981; Johnson, 1999).

Binding studies of NOP-1 at tomato receptors LeETR4 and NR by microscale thermophoresis

Binding of the NOP-1 octapeptide to purified recombinant tomato receptors LeETR4 and NR was analysed by microscale thermophoresis (MST) (Duhr and Braun, 2006; Wienken et al., 2010; Jerabek-Willemsen et al., 2011; Seidel et al., 2013). Receptors (100 nM) labeled with Alexa Fluor 488-Maleimide (Thermo Fisher Scientific) were titrated with peptide ligand NOP-1 dissolved in 50 mM Tris-HCl pH 8.0, 300 mM NaCl at concentrations from 500 µM to 61.04 nM. Then, samples were transferred into standard glass capillaries and thermophoresis was measured using a Monolith NT.115 (NanoTemper Technologies GmbH, München, Germany). MST measurements were recorded at 20% MST power for LeETR4-NOP1 and 60% MST power for NR-NOP-1, respectively. Receptors were chemically denatured by incubation with the strong ionic detergent SDS (4% (w/v)) and the small-molecule redox reagent DTT (40 mM) for 5 min in the dark at RT and served as control to confirm specific and selective binding of the ligand. All measurements were run in triplicate.

Fruit material, treatments and storage conditions

Tomato fruits (*Solanum lycopersicum L.*) of the cultivar ‘Lyterno’ (Rijk Zwaan, De Lier, Netherlands) were harvested at the maturity stage “green” (USDA; 2005) from tomato plants which where cultivated in a commercial-like greenhouse at the research station Campus Klein-Altendorf (University of Bonn, Germany). At the early development stages, the trusses were manually thinned to six fruits per truss, according to the common practice aiming standardized fruit size and quality. For the experiment, the last two fruits

of the fourth truss, counted from the bottom, were chosen for evaluations. At the beginning of the experiment, 150 fruits showing similar color and size were divided into four treatments (n = 25 fruits per treatment). On each fruit, a transparent polyethylene film was placed at the equatorial zone to demark the four evaluation points of 2.8 cm diameter each.

The four treatments were as follows: (1) control; (2) NOP-1, 400 µM; (3) NOP-1, 1000 µM; (4) NOP-1, 2000 µM. The NOP-1 peptide (GenScript, Piscataway, NJ, United States) was dissolved in 5 ml deionized water. On each fruit, a total of 200 microdroplets (0.5 µL each) of the peptide solution were gently deposited (50 micro droplets on each marked area of the tomato fruit) with a Hamilton microdispenser (Hamilton Bonaduz AG, Bonaduz, Switzerland). Fruits of the control treatment received an equal number of microdroplets of deionized water. After application of the droplets fruits of each treatment were allocated in storage boxes thereby avoiding fruit-to-fruit contact and stored at room temperature (19 ± 2°C).

Non-destructive measurements of fruit color at ripening

Starting at the beginning of the experiment until 24 days after treatments (DAT), fruit ripening was evaluated and monitored twice a week with two non-destructive sensors using the principles of light reflectance and fluorescence emission. Evaluations were done on the marked fruit zones (n = 4 areas/fruit, n = 25 fruits per treatment).

Changes of the surface color over time were determined with a portable spectrophotometer (CM-503d, Konica Minolta Inc., Tokyo, Japan), which has a sensing area of 7 mm^2. Based on the CIELAB model (McGuire, 1992), the recorded parameters are converted into the hue° index. Hue was calculated according to the following formula:

$$hue° = tan^{-1}\left(\frac{b*}{a*}\right),$$

where ‘a*’ and ‘b*’ are defined as color coordinates, provided that ‘a*’ is the point of the green-red axis and ‘b*’ is defined as the point of the yellow-blue axis. On the basis of the above, the resulting angle is converted into the corresponding color value.

Fluorescence based analysis of fruit maturity

Pigment fluorescence was applied as second non-destructive technique to address fruit

maturation. To this end, a handheld device (MultiplexR 3, Force-A, Orsay, France) equipped with light-emitting diodes (LEDs) with UV (375 nm), blue (475 nm), green (510 nm) and red (635 nm) excitation was used. Fluorescence was detected in the blue-green (BGF, 425–475 nm), red (RF, 680–690 nm) and near-infrared (FRF, 720–755 nm) spectral regions (Ben Ghozlen et al., 2010). Based on the absolute fluorescence signals recorded in a detection diameter of approximately 2 cm, simple and complex fluorescence ratios were calculated. The parameter Simple Fluorescence Ratio excited with red light (SFR_R) as a measure of the chlorophyll content is as follows:

$SFR\_R = (\frac{FRF\_R}{RF\_R})$ (according to Ben Gholzen et al., 2010).

Determination of chlorophyll, ß-carotene and lycopene

Determination of the pigment content in the fruits was done weekly on five fruits each treatment. The concentrations of ß-carotene, lycopene and chlorophyll were analyzed from freeze-dried and ground fruit samples, as described below. Concentrations of chlorophyll, ß-carotene and lycopene were analyzed according to the method of Nagata and Yamashita (1992) as described by Barros et al. (2007), Azeez et al. (2012) and Kalogeropoulos et al. (2012) with the following modifications. Briefly, 1.5 ml of the solvent Aceton: Hexane (4:6) was added to 0.1 g of the freeze-dried and ground material, homogenized and centrifuged for 10 min at 16,100 × g (CENTRIFUGE 5415 R, Eppendorf AG, Hamburg, Germany). Next, 2 ml of the solvent was added to the supernatant and the absorption of the solutions was determined at 453, 505, 645, and 663 nm in a LAMBDA 35 spectrophotometer (Perkin Elmer R, Waltham, MA, United States). Concentrations of chlorophyll, ß-carotene and lycopene were calculated according to Nagata and Yamashita (1992) by the following equations:

$$Chlorophyll\ a\ [mg\ 100ml] = 0.999A_{663} - 0.0989A_{645}$$

$$Chlorophyll\ b\ [mg\ 100ml] = -0.328A_{663} + 1.77A_{645}$$

$$ß - carotene\ [mg\ 100ml] = 0.216A_{663} - 1.22A_{645} - 0.304A_{505} - 0.452A_{453}$$

$$Lycopene\ [mg\ 100ml] = -0.0458A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453}$$

Total chlorophyll was calculated by adding chlorophyll a and chlorophyll b content.

Statistical analysis

All results are expressed as mean ± SE. Analyses of variance were determined with one-way ANOVA ($a \leq 0.05$). In case of statistical significance, the Tukey's HSD ($\alpha \leq 0.05$) was applied to establish the differences among means. Statistical analyses were carried out using SPSS 22.0.

3. Results

Expression and purification of tomato receptors LeETR4 and NR

Codon optimized synthetic genes encoding full-length ethylene receptors LeETR4 and NR were each cloned into expression vector pET16b by Gibson Assembly Cloning (Gibson et al., 2009). Expression vectors encoding the tomato receptors were transformed into cells of *E. coli* strains BL21 (DE3) and C43 (DE3) which have been successfully applied for the expression of different members of the ethylene receptor family from *A. thaliana, Lycopersicon esculentum* and *Physcomitrella patens* in previous studies (Voet-van-Vormizeele and Groth, 2008; Classen and Groth, 2012). Protein expression was induced by the addition of 0.5 mM IPTG. Optimum expression was obtained for LeETR4 after 20 h in C43 (DE3) at 16°C, while systematic analysis of expression parameter for NR showed best expression after 6 h in BL21 (DE3) at 16°C (Fig. 1).

Receptors were localized in the membrane fractions of the host and solubilized from these membranes by the mild detergent Fos-Choline-16. After solubilization, receptors were purified in a single chromatography step on Ni–NTA agarose (GE Healthcare Life Sciences, Munich, Germany). Purification of the recombinant tomato receptors was analyzed by SDS–PAGE. The related protein gels (Fig. 2) show prominent bands at 90 and 70 kDa corresponding to the molecular weight of LeETR4 (88 kDa) and NR (74 kDa). Besides, two minor contaminations were detected in the lower MW range at 55 and 32 kDa, respectively. Hence, both receptors have been successfully purified from their heterologous host. Identity of the receptors was confirmed by antibodies directed against the deca-histidine tag in both proteins.

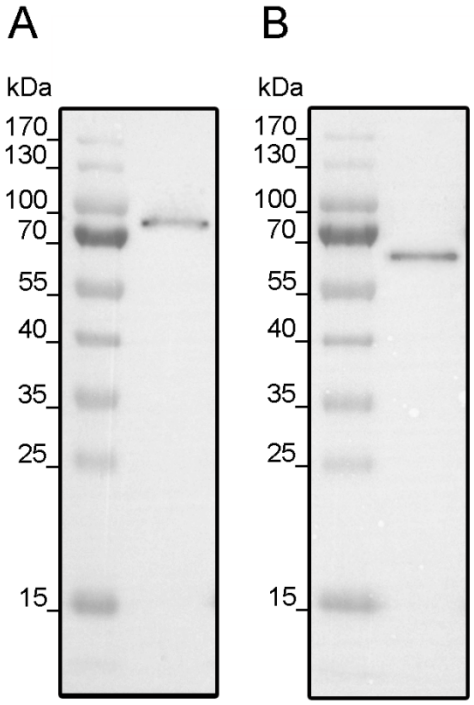

Figure 1. Expression of LeETR4 (A) and NR (B). (A) *E. coli* C43 (DE3) was transformed with pET16b_LeETR4 and expressed for 20 h after induction at 16 °C. (B) pET16b_NR was transformed into *E. coli* BL21 (DE3) and expressed for 6 h after induction at 16 °C. For both expressions, host cell extract was analyzed by Western blotting using an Anti-His antibody targeting deca-histidine tagged proteins.

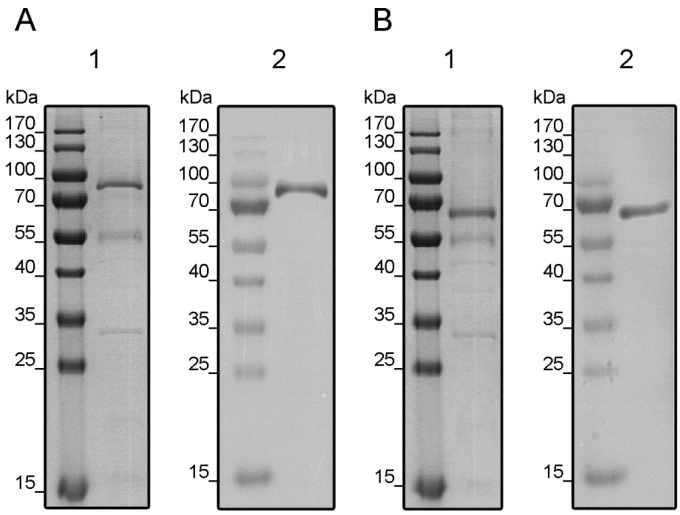

Figure 2. Purification of solubilized and His-tagged LeETR4 (A) and NR (B) by IMAC. Purified proteins LeETR4 and NR were analyzed by SDS-PAGE. Purified proteins were visualized by colloidal Coomassie staining (1) and Western blotting (2) using an anti-His antibody.

Secondary protein structure and functional folding of purified LeETR4 and NR

Folding and protein secondary structure of purified recombinant tomato receptors were probed by CD spectroscopy. The corresponding spectra of LeETR4 and NR shown in Figure 3 are typical of partially helical proteins, displaying two minima at approximately

209 and 222 nm with an isosbestic point at 202 nm. Overall, the spectra of both receptor proteins are highly similar and correspond to previous CD data on receptor orthologs from *Arabidopsis* and *Physcomitrella* (Classen and Groth, 2012). Secondary structure calculations by CDSSTR and CONTINLL suggest an α-helix content of 34% and a ß-sheet percentage of 18–20% for LeETR4. Similar numbers of 41– 42% α-helix and 14% ß-sheet structure were obtained for NR. Consequently, CD spectroscopic measurements verify that the purified receptors adopt a well-folded structure and are indicative for a native conformation of the recombinant tomato proteins.

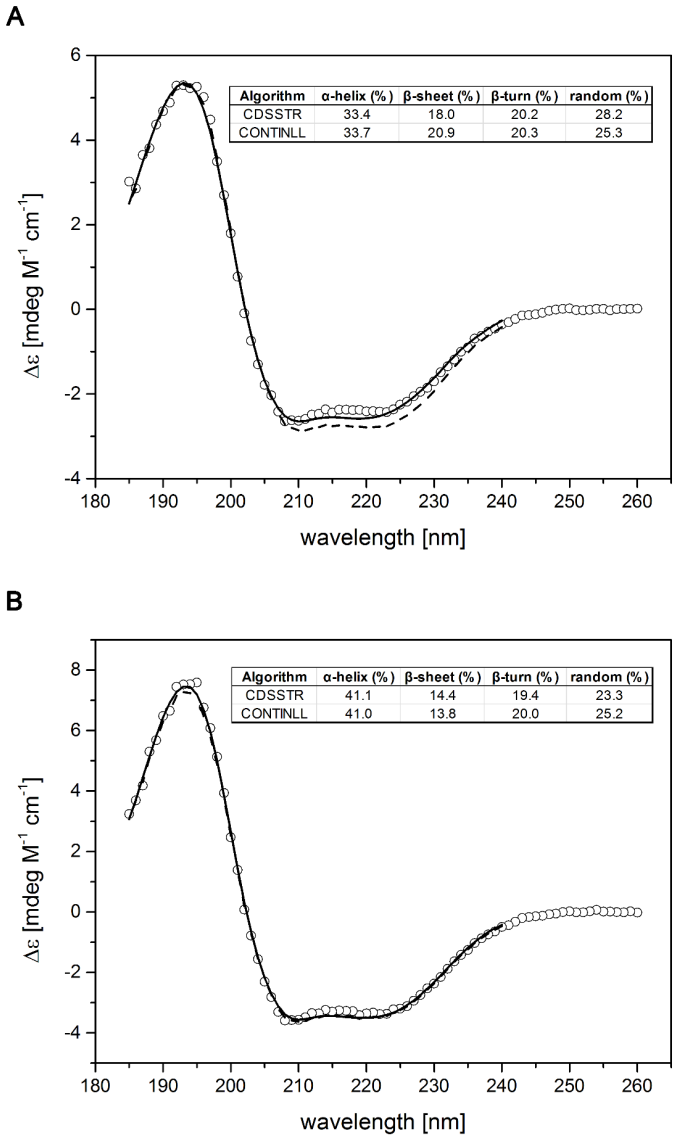

Figure 3. Experimental and calculated CD spectra of purified LeETR4 (A) and NR (B). The far-UV CD spectra of LeETR4 and NR (○) were obtained by accumulating ten spectra with 1nm bandwidth and a scanning speed of 50 nm min^{-1}. The CD data were adjusted to molar extinction (Δε) considering molecular weight and protein concentration of the ethylene receptors. The secondary structure calculations were determined by using the CDSSTR (dashed line) and CONTINLL (solid line) program (Provencher and Gloeckner, 1981; Johnson, 1999).

Binding of NOP-1 to purified recombinant tomato receptors LeETR4 and NR

Analysis of protein–ligand interactions by MST was used to monitor and to quantify the interaction of synthetic NOP-1 octapeptide with purified recombinant LeETR4 and NR, respectively. Ligand binding and the related dissociation constant with the isolated receptors were deduced from changes in thermophoresis upon addition of the NOP-1 octapeptide (Fig. 4). In analogy to previous studies on receptors from *Arabidopsis* and constitutively expressed tomato receptor LeETR1 (Bisson et al., 2016) clear changes of the thermophoretic signal were observed upon addition of the synthetic peptide with purified recombinant LeETR4 and NR receptors that are expressed at high levels at fruit ripening. Selectivity of the peptide–receptor interaction was probed in MST studies with chemically denatured receptor proteins. In these experiments, no change in thermophoresis was detected upon addition of NOP- 1 (Fig. 4). The apparent dissociation constant (K_d) calculated from the changes in thermophoresis induced by different amounts of NOP-1 added to fluorescently labeled tomato receptors was 4.15 ± 0.85 µM for LeETR4 and 23.52 ± 1.99 µM for NR, respectively (Fig. 4). Both numbers are in the lower micromolar range and together with the negative controls on denatured receptor proteins are indicative of efficient and specific binding of NOP-1 to tomato receptors LeETR4 and NR.

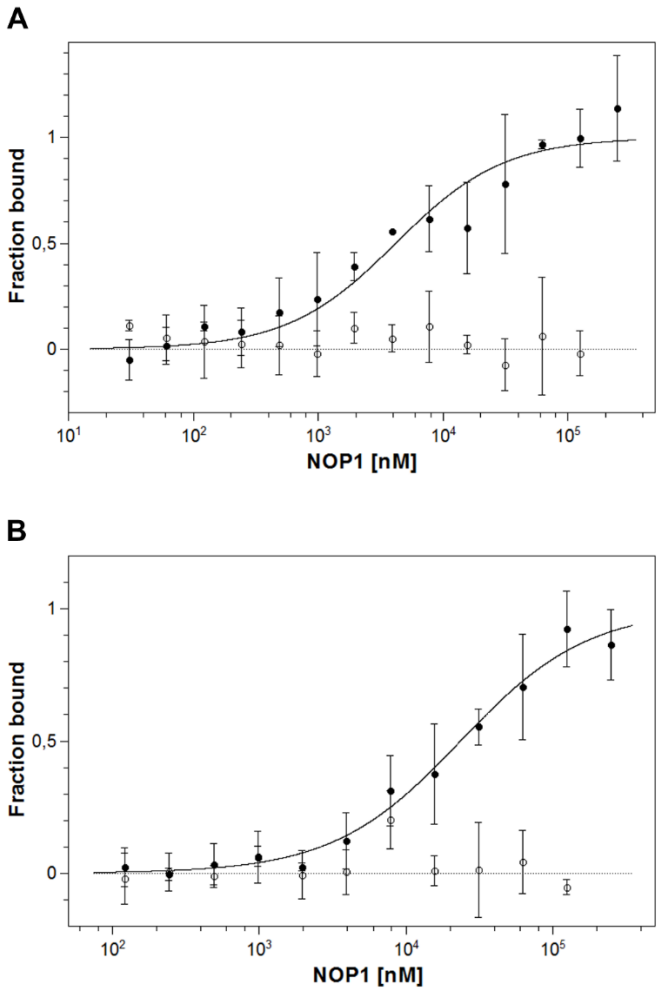

Figure 4. Microscale thermophoresis (MST) interaction studies of tomato ethylene receptors to NOP-1. (A) Determination of K_d value of unlabeled NOP-1 peptide to LeETR4 (●) based on MST is shown. A K_d value of 4.15 µM ± 0.85 µM was obtained. A negative control using chemically denatured LeETR4 (○) shows no further interaction with binding partner NOP-1. (B) Calculation of K_d value of the small peptide NOP-1 to NR (●) using MST technology resulted in a K_d value of 23.52 µM ± 1.99 µM. Chemically denatured NR (○) indicates no further binding event with NOP-1. All data represent the mean of three independent measurements ± standard deviation.

Impact of NOP-1 on fruit ripening and fruit quality

For many fruits and vegetables color development is the most important external characteristic to assess ripeness and postharvest life. Color change from green to red was slowed down in tomato fruits treated with NOP-1 (1000 µM), as indicated by the hue° index which was significantly higher on DAT 4, 7, and 9 in this treatment group as compared to control (untreated) fruits (Fig. 5A). For treatment with 400 or 2000 µM NOP-1 slower color change as compared to control fruits were observed only at DAT 4, and to a smaller extent at DAT 7. Thereafter, color change of treated fruits was similar to controls. From DAT 14 onwards, there were no significant differences in hue°among all evaluated treatments. Similar to the observed effects on the hue index, application of 1000 µM NOP-1 also resulted in a significantly higher Simple Fluorescence Index

(SFR_R, estimating chlorophyll concentration) on DAT 4, 7, and 9 (Fig. 5B). A slight increase in SFR_R compared to non-treated fruits was further observed for fruits treated with 400 and 2000 µM NOP-1 at DAT 7. Finally, all treatments reached similar values on DAT 14 and no further changes were observed until the end of the experiment (DAT 23).

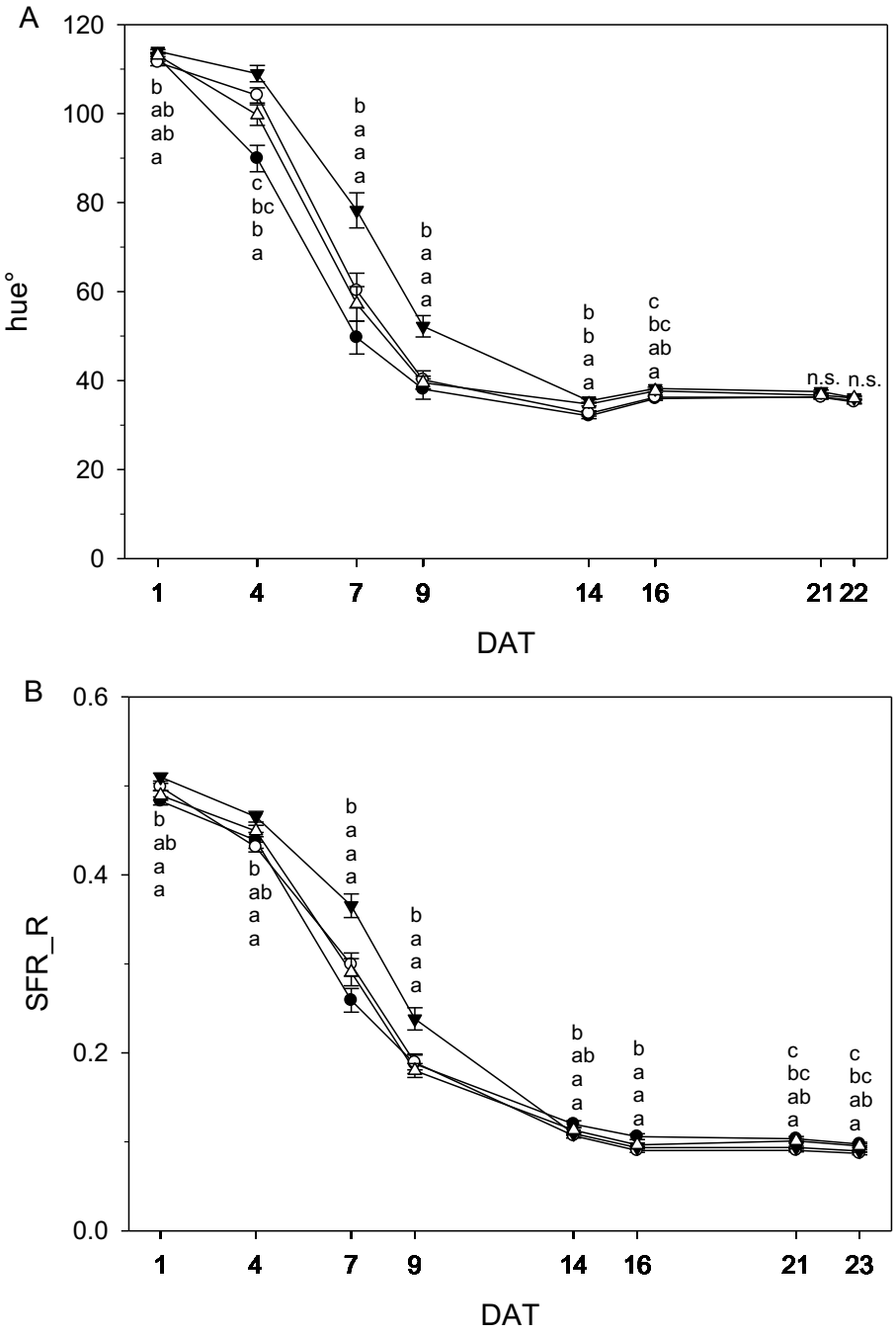

Figure 5. Hue° index (A) and SFR_R index (B) of control tomato fruits (●) and tomato fruits treated with NOP-1 400 µM (○), 1000 µM (▼) or 2000 µM (Δ). Data are means ± SE, n = 3×25, - 5 each week, different letters indicate significant differences between treatments on each measurement day (Tukey´s HSD, $\alpha \leq 0.05$).

Color development is another sensitive but invasive measure to monitor ripening. The chlorophyll content of tomato fruits over time is shown in Figure 6A. Fruits treated with 1000 µM NOP-1 showed higher concentrations of total chlorophyll compared to non-treated controls throughout the experiment. While total chlorophyll decreased rapidly to about 30% after DAT 9 in controls, only 50% of the pigment originally present was degraded in fruits treated with 1000 µM at this time. However, over time chlorophyll breakdown in fruits treated with 1000 µM NOP-1 converged to chlorophyll degradation in controls and had essentially ceased after DAT 16. Surprisingly, fruits treated with 400 and 2000 µM NOP- 1 showed a more pronounced chlorophyll breakdown on DAT 9 than non-treated controls. However, with further progression of the experiment chlorophyll degradation in these fruits ceased and chlorophyll levels adapted to controls on DAT 16–24.

In contrast to the observed degradation in chlorophyll concentration of ß-carotene increased in all treatments throughout the experiment (Fig. 6B). However, fruits treated with 1000 µM NOP-1 showed a slower increase in ß-carotene concentration and revealed significantly lower levels of this pigment at DAT 9 when compared to all other treatments which essentially showed the same pattern for the increase of this carotenoid during ripening. Over time ß-carotene in the fruits treated with 1000 µM NOP-1 increased to control levels. All treatments showed comparable levels of this carotene on DAT 16 and concentration of this pigment remained constant throughout the further experiment. Slightly higher concentrations of ß-carotene were observed for the 400 µM treatment at DAT 23–24.

The concentration of lycopene principally responsible for the characteristic deep-red color of ripe tomato fruits, increased significantly in all treatment groups (Fig. 6C). Similar to the pattern observed for ß-carotene, treatment with 1000 µM NOP-1 showed the largest delay in pigment accumulation at DAT 9 where only 33% of the lycopene level of non-treated controls was measured. Treatment with 400 µM of the synthetic peptide still resulted in a delay of lycopene accumulation of about 69% compared to non-treated controls. According to the pattern observed with ß-carotene, lycopene levels in all treatments adjusted to similar concentrations after DAT 16 and stayed constant for the rest of the experiment. Highest concentrations of lycopene were measured for all treatments in completely ripe tomatoes at DAT 24. Total color development of fruits treated at different concentrations of NOP-1 is illustrated by visual images of whole fruits (Fig.7).

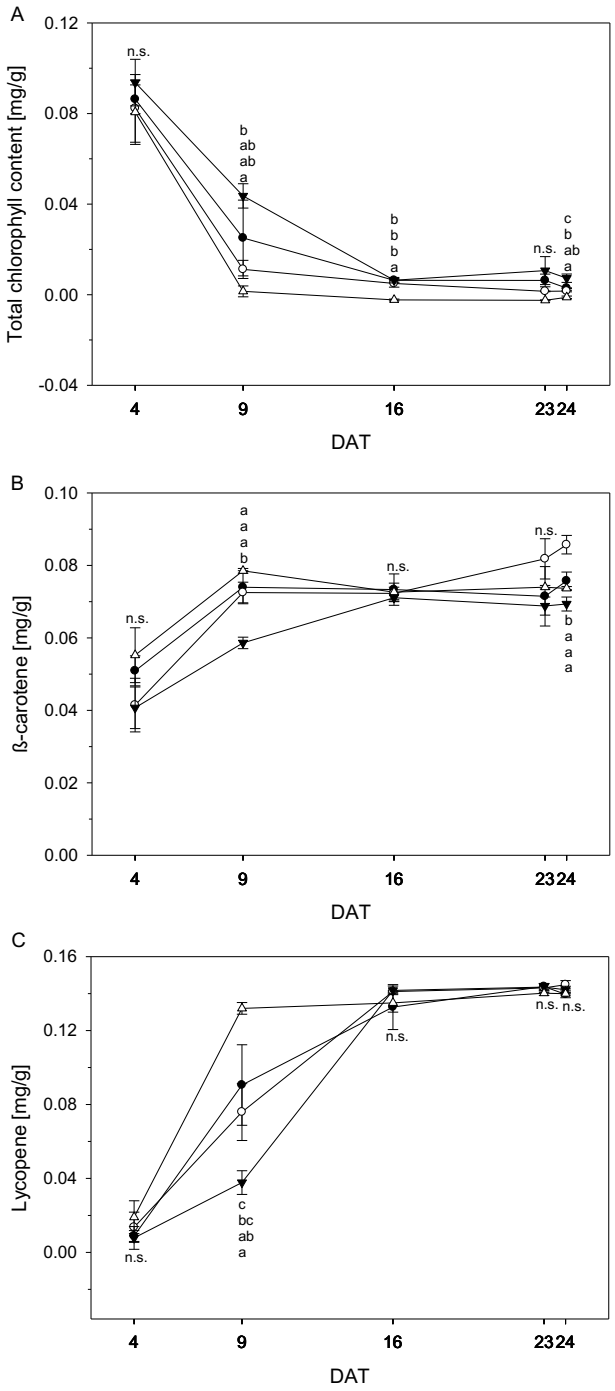

Figure 6. (A) Total chlorophyll content of control tomato fruits (●) and tomato fruits treated with NOP-1 400 µM (○), 1000 µM (▼) or 2000 µM (Δ). Concentration of ß-carotene (B) and lycopene (C) of control tomato fruits (●) and tomato fruits treated with NOP-1 400 µM (○), 1000 µM (▼) or 2000 µM (Δ). Data are means ± SE, n = 5, different letters indicate significant differences between treatments on each measurement day (Tukey´s HSD, $\alpha \leq 0.05$).

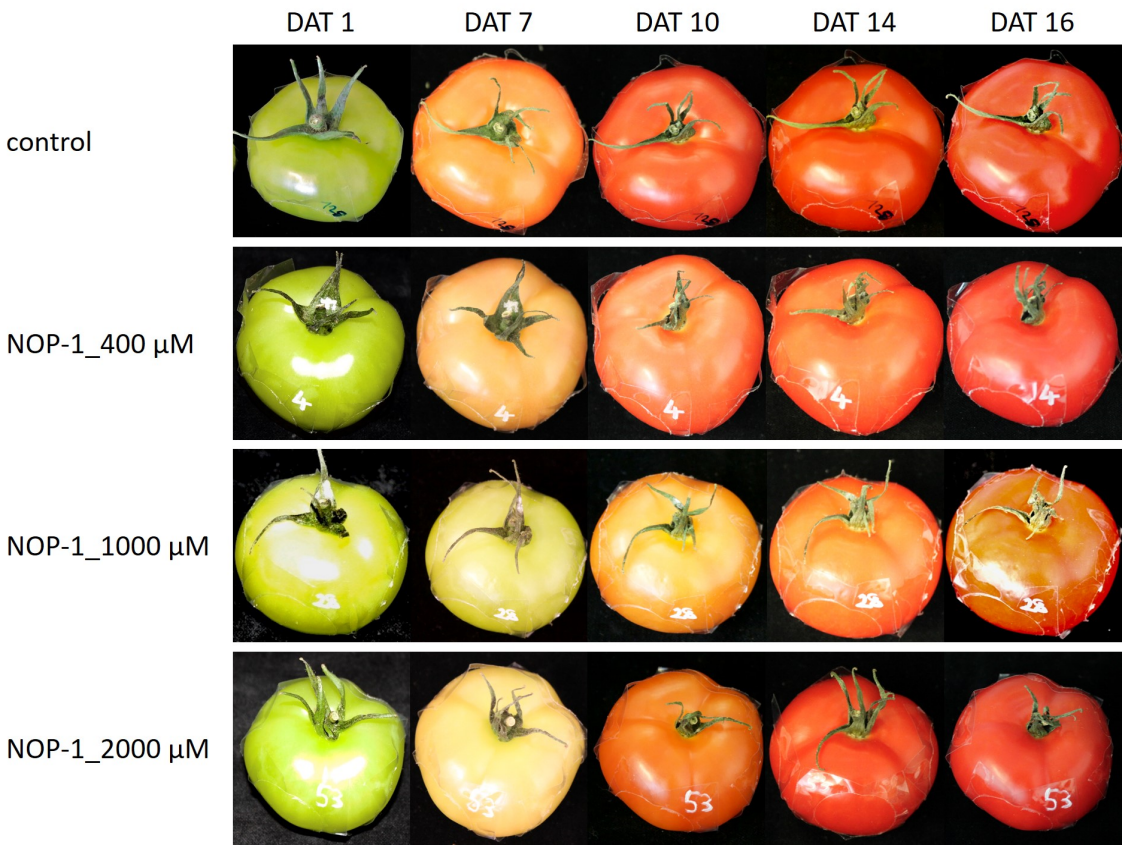

Figure 7. Visual images of fruits treated at different concentrations of NOP-1. Representative photos of whole fruits treated with 400, 1000 and 2000 µM NOP-1 on DAT 1, 7, 10, 14, and 16. Control fruits are depicted in the upper row.

Fruit soften during ripening due to biochemical processes resulting in the breakdown of cell-wall polymers. Hence, firmness is an indirect measurement of ripeness and represents one of the most important variables for fruit quality. Consequently, we determined fruit firmness in tomato fruits treated with NOP-1 and non-treated controls using a non-destructive sensor and a shore scale ranging from 0 to 100 units. Firmness decreased from 80–89 shore to 55–50 shore in the course of the experiment (Fig. 8). Firmness fruits were observed at the beginning of the experiment, softest fruits were measured at the end. Control, treatment with NOP-1 at concentrations of 400 and 2000 µM showed a continuous decrease in fruit firmness over time, whereas firmness was unaffected at early stages (DAT 4–9) in fruits treated with 1000 µM NOP-1. However, after the initial lag phase firmness decreased to similar levels in these fruits as observed for the other treatments on DAT 16 and later stages. All treatments showed similar numbers for fruit firmness and thereby comparable fruit quality at the end of the experiment on DAT 24.

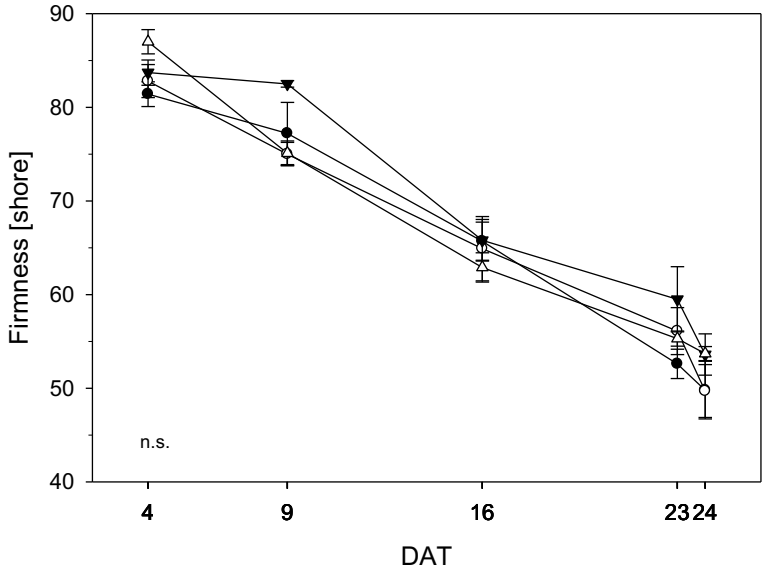

Figure 8. Fruit firmness of control tomato fruits (●) and tomato fruits treated with NOP-1 400 µM (○), 1000 µM (▼) or 2000 µM (Δ). Data are means ± SE, n = 5. Statistical analysis according to Tukey´s HSD ($\alpha \leq 0.05$) revealed no significant (n.s.) difference in all treatments.

4. Discussion

Previous studies on *Arabidopsis* demonstrated that the small basic peptide NOP-1 derived from the natural NLS-sequence of the ethylene regulator protein EIN2 is able to disrupt ethylene signalling and inhibit plant ethylene responses. Protein–protein interaction studies on recombinant purified proteins EIN2 and ETR1 and related FRET studies in planta suggest that the inhibitory peptide competes for binding of EIN2 at the receptors offering a novel way to interfere with ethylene signal transduction and ethylene responses in planta (Bisson and Groth, 2015; Bisson et al., 2016). The high conservation of the NLS-motif among the plant kingdom (see Supplementary Fig. S1 in Bisson et al., 2016) and the level of homology in ethylene receptors open up new avenues for ripening control of fruits and vegetables by biological peptides in modern agriculture and horticulture. Initial studies on tomato, a climacteric fruit of high economic and nutritional impact serving as model to study fruit ripening, support these ideas and confirm the results obtained with the *Arabidopsis* genetic model.

In order to further evaluate the potential of the inhibitory peptide identified in previous studies, we have further analyzed molecular and physiological effects of the basic NLS-derived peptide NOP-1 on tomato. Our studies with purified recombinant receptors LeETR4 and NR, which are both highly expressed in ripening fruit, reveal efficient binding of the peptide to both receptors and thereby confirm that the NOP-1 octapeptide

may interact with receptors from both receptor subfamilies. Both receptors interact with the peptide at affinities in the lower µM-range, but interaction with LeETR4 seems to be stronger.

Noteworthy, previous studies demonstrate an even stronger binding affinity of NOP-1 at tomato receptor LeETR1 – a receptor of subfamily I continuously expressed throughout the plant. However, LeETR4 and NR are quite different from LeETR1 with respect to kinase activity as well as in the number and their type of phosphorylation sites (Kamiyoshihara et al., 2012). Keeping further in mind the actual sequence identities of *Arabidopsis* ETR1 and tomato LeETR1/NR/LeETR4 of 81, 69, and 41%, the observed range in affinities of different receptors and receptor subfamilies for NOP-1 is not surprising at all. Bearing in mind that NOP-1 was derived from the NLS motif in EIN2 the different binding affinities observed with the peptide may also suggest that receptors have different affinities for the EIN2 central hub.

In associated post-harvest studies, we have evaluated changes in color and texture development of tomato fruits treated with different concentrations of NOP-1 and the impact of the NLS-derived peptide on fruit ripening and fruit quality. Our studies show clear effects on fruit ripening at concentrations of 1000 µM, whereas effects on color development at 400 µM are substantially less pronounced and manifest only at early ripening stages (DAT 9 – see Fig. 6C). In total, a concentration of 400 µM NOP-1 applied to the fruit surface as microdroplets seems to be too low for significant inhibition of the ethylene transduction cascade, whereas 1000 µM allows a ripening delay, as expressed by the chlorophyll degradation and lycopene and ß-carotene accumulation (Fig. 6). When applied at 2000 µM concentration no effect of NOP-1 was observed which might be related to concentration-dependent aggregation or changes in secondary structure (Garg et al., 2013), which may impair uptake of the peptide by the fruit surface. Alternatively, the fact that no significant positive effect on ripening delay was observed at 2000 µM concentration in contrast to 1000 µM NOP-1 may be explained by differences in droplet–surface–uptake interactions at the different concentrations. In this case, the solution concentration of 1000 µM showed higher uptake of NOP-1 at the same contact surface area (i.e., tomato fruit surfaces), possibly due to the optimum dose/concentration/interfacial area which apparently causes the maximum penetration. For 2000 µM droplet the NOP-1 loading was twice as high at the same water volume. Consequently, this may have reduced the droplet viscosity on the cuticle, also apparent by its modified macroscopic appearance and altered biomechanical properties

(Domínguez et al., 2011; Burkhardt and Hunsche, 2013). In consequence, penetration and uptake of NOP1 may have been reduced, resulting in a less effective 2000 µM treatment.

Improving shelf-life and nutritional quality of tomato fruits is difficult to achieve with the methods currently in use. Maintaining adequate storage conditions is expensive and might cause chilling injury if used improperly (Passam et al., 2007). Even though genetic modifications reducing gene expression of proteins involved in ethylene synthesis are possible in principle (Abano and Buah, 2015) these procedures are banned by law throughout Europe and have low acceptance among European consumers. Chemical methods such as application of AVG to inhibit ethylene biosynthesis or treatment with 1-MCP to inhibit ethylene response significantly delay ripening and slow down lycopene synthesis and chlorophyll breakdown (Saltveit, 2005; Passam et al., 2007). The drawbacks of these methods correlating with the restricted use of both chemicals on tomatoes in Europe also relate to quality losses in taste development or to complete arrest in maturation, as observed in some cases (Passam et al., 2007).

In summary, our study shows that the NOP-1 octapeptide derived from the NLS-motif at the EIN2 C-terminus is a potent inhibitor of the maturation process in tomato. The peptide efficiently binds to different receptor isoforms and, when applied to the surface of immature fruit, successfully delays the ripening process without impairment of final overall fruit quality at the fully mature stage. This novel approach to delay fruit ripening is making use of a synthetic peptide that corresponds to the highly conserved NLS-motif in all known EIN2 sequences and holds great promise to control processes such as ripening or senescence in horticultural and agricultural applications.

5. Author contributions

GG conceived the project. GG, GN, and MH planned, designed and supervised the research. MK, SK, and LM performed the experiments and contributed equally to this work. All authors contributed to data analysis and the writing of the manuscript.

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C Influence of the ethylene-related signal-inhibiting octapeptide NOP-1 on postharvest ripening and quality of 'Golden Delicious' apples[2]

1. Introduction

Half of the plant food produced for human consumption does not reach the consumer, with about 50% of those food losses being vegetables and fruits (Blanke, 2014). One important reason for the quantity and quality of postharvest losses is the ripening process of climacteric fruits.

The maturity development of climacteric fruit is controlled by the plant hormone ethylene (Abano and Buah, 2015). Via activation of ethylene receptors at the endoplasmic reticulum membrane, a signalling cascade leads to an activation of ripening-related genes (Abano and Buah, 2015; Kessenbrock et al., 2017). This induces changes in fruit colour, flesh firmness, aroma and texture as chlorophyll, cellulose and pectin are broken down and carotenoids and aroma volatiles are produced (Alexander and Grierson, 2002; Brady, 1987; Bruyn and van Keulen, 1968). However, there is also an ethylene-independent regulation of climacteric fruit ripening, where the loss of acidity, accumulation of sugars and the initiation of the climacteric process do not require ethylene (Pech et al., 2008). The ripening process in climacteric fruits is also accompanied by changes in cell respiration with increased carbon dioxide (CO_2) production (Hulme et al., 1963).

To reduce the postharvest spoilage of climacteric fruits, in addition to control of temperature and use of controlled atmospheres, ripening might be slowed down or delayed with ethylene synthesis inhibitors. Treatments with 1-methylcyclopropene (1-MCP) inhibit ethylene receptors and prevent the ethylene response cascade (Blankenship and Dole, 2003). Through this, ethylene release is also strongly decreased due to the inhibition of the autocatalytic ethylene synthesis (Lelievre et al., 1997; Yang et al., 2013). Other methods to delay ripening include the use of the enzyme inhibitor

[2]Klein, S., Fiebig, A., Neuwald, D., Dluhosch, D., Müller, L., Groth, G., Noga, G., Hunsche, M., (2019): Influence of the ethylene-related signal-inhibiting octapeptide NOP-1 on postharvest ripening and quality of 'Golden Delicious' apples. In: *Journal of the Science of Food and Agriculture 99 (8),* 3903-3909.

aminoethoxyvinylglycine, which blocks 1-aminocyclopropane-1-carboxylate synthase (Zhu et al., 2008) or the application of ethylene antagonists such as cobalt(II) ions, or genetic engineering techniques (Lau and Yang, 1976; Ayub et al., 1996; Lau and Yang, 1976). However, these methods are often limited due to difficulties in handling, the high technical effort required, doubts over the safe use of genetic engineering or toxicity.

New insights about the ethylene signalling pathway may allow a better control of ethylene-induced ripening effects by means of oligopeptides (NOP-1) derived from the nuclear localization signal (NLS) at the C-terminal end of the ethylene signalling protein ethylene-insensitive-2 (EIN2), which interferes with a protein–protein interaction (Bisson and Groth, 2011; Bisson et al., 2016). The synthetic peptide NOP-1 (LKRYKRRL) inhibits the interaction of the ethylene receptor ETR1 and EIN2. NOP-1 has been shown to be effective in delaying the ripening process in tomato without fruit quality impairment (Bisson et al., 2016; Kessenbrock et al., 2017). The NLS-motif and the ETR are highly conserved in the plant kingdom (Bisson and Groth, 2011). Consequently, NOP-1 seems to be a promising opportunity to investigate the control of ripening in other climacteric fruit, such as apples (Kessenbrock et al., 2017).

To explore the efficacy of NOP-1 for ripening control in apples, we first tested binding of NOP-1 to the isolated purified ethylene receptor MdETR1 from *Malus domestica*, which shares about 42–84% sequence identity with the corresponding tomato receptors LeETR4, NR and LeETR1 (Kessenbrock et al., 2017).

It is unclear how the peptide penetrates through the fruit skin and moves from cell to cell. For a practice-oriented treatment, it is important to compare different application techniques. Therefore, this study evaluated the efficiency of NOP-1 on apple to inhibit fruit ripening in comparison with a 1-MCP treatment. Two different surface application methods were tested on ‘Golden Delicious’ apples, and fruit quality parameters were assessed. To determine the ripening development of apple fruits, non-destructive and destructive measurements were used.

2. Material and methods

Experiment 1: expression and purification of the recombinant apple ethylene receptor MdETR1

For the expression of MdETR1, the corresponding expression vector (pET-16b) was transformed into *Escherichia coli* strain BL21 (DE3). Cultures were grown in 2YT-media [1.6% (w/v) peptone, 1% (w/v) yeast extract and 0.5% (w/v) sodium chloride (NaCl)] in the presence of 2% (v/v) ethanol and 100 µgmL^{-1} ampicillin at 37°C. At OD_{600} =0.5–0.6, the temperature was reduced to 16°C and expression was induced at OD_{600} =0.8 by the addition of 0.5 mmol L^{-1} isopropyl-β-D-1-thiogalactopyranoside. Cells were harvested 20 h after induction by centrifugation for 15 min at 7000×g and 4°C.

The soluble C-terminal domain of AtEIN2 (aa 479–1294) was expressed and purified analogous to its tomato homologue as described by Bisson et al. 2016. For the purification of the apple ethylene receptor MdETR1, a purification protocol described by Kessenbrock et al. 2017 was adopted and modified for the solubilization. Membrane pellets were solubilized in solubilization buffer [200 mmol L^{-1} NaCl, 50 mmol L^{-1} Tris hydrochloride (Tris/HCl) pH 8, 1% (w/v) Fos-Choline-16, 0.002% (w/v) phenylmethylsulfonyl fluoride] for 1 h at 700 rpm and 4°C. Expression and purification of recombinant proteins were examined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on 12% SDS gels and Coomassie staining as well as western-blotting analysis for identification of target proteins.

Experiment 2: binding of NOP-1 to purified MdETR1

Interaction of the purified apple ethylene receptor with EIN2 and NOP-1 octapeptide (GenScript, Piscataway, NJ, USA; lot no. 92436550001/PE0892) was studied by microscale thermophoresis (MST). The receptor was labelled using Alexa Fluor 488 succinimidyl ester (Thermo Fisher Scientific, Waltham, MA, USA). For MST measurements, 50 nmol L^{-1} of the receptor was titrated with either EIN2 in concentrations ranging from 2 µmol L^{-1} to 0.49 nmol L^{-1} or NOP-1 in concentrations ranging from 250 µmol L^{-1} to 61.04 nmol L^{-1} in MST buffer [300 mmol L^{-1} NaCl, 50 mmol L−1 Tris/HCl pH 7.8, 5% (v/v) glycerol and 0.0075% (w/v) Fos-Choline-16]. Negative controls were carried out by chemical denaturation of proteins using 40 mmol L^{-1} dithiothreitol, 2% (w/v) SDS and incubation for 5 min in the dark. For measurements with NOP-1, 50 mmol L^{-1} phosphate buffer was used instead of Tris/HCl. Afterwards,

samples were transferred into standard glass capillaries and measurements took place using a Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany) at 60% MST power. All measurements were performed in triplicate.

Experiment 3: effect of NOP-1 on the ripening development and fruit quality of apple

Fruit, treatments and storage conditions

Apples (*M. domestica*) cv Golden Delicious were harvested from mature trees at the research station Campus Klein-Altendorf (University of Bonn, Meckenheim, Germany). The harvest date (11 September 2017) was selected in accordance with standard commercial harvest maturity (fruit flesh firmness: 71.6 N; starch index: 6.3; soluble solids: 11.48; acidity: 0.62). After harvest, fruit were stored at 1°C for 1 day before being allocated into experimental treatments.

For the first part of the experiment, fruit were randomized, and homogeneous apples were allocated into the experimental treatments (n=15 fruits). The following treatments were applied: control (untreated), NOP-1_1000 µmol L^{-1} applied with a microdispenser (Hamilton Bonaduz AG, Bonaduz, Switzerland; 700 s Series; fixed volume 25 µL; repeated kit valve), NOP-1_1000µmol L^{-1} applied with brush, and 1-MCP (1000 nL L^{-1}). The NOP-1 peptides were dissolved in deionized water. For the microdispenser treatments, 320 microdroplets (0.5 µL each) of the peptide solution were applied on each apple fruit; for the brush treatment, 300 µL of the peptide solution was spread equally on each fruit with a fully saturated brush. A concentration of 1000 nL L^{-1} (1.6 gm^{-3} of SmartFresh™ 0.14% active substance) in air was set for the 1-MCP treatment (SmartFresh, Agrofresh, Milan, Italy), while the application was performed according to the manufacturer instructions for 24 h in a polypropylene container. The tailor-made cabinets (about 0.40 × 0.40 × 0.60m^3) were made of transparent Plexiglas and properly sealed at the borders to ensure no gaseous exchange with the external environment. After treatment, apples were stored for 41 days in 70 L polypropylene boxes at 6°C and relative humidity of 97% followed by a 2-week shelf-life (19.5±0.5 ∘C; relative humidity 55±0.5%).

Non-destructive measurements

Two optical sensors, a Minolta portable spectrophotometer (CM-700d, Konica Minolta Inc., Tokyo, Japan) and a handheld Multiplex® 3 (Force-A, Orsay, France) multi-parametric optical fluorescence sensor were used to determine apple ripening over the trial period. Determinations were done at three points around the equatorial zone of the fruit. Skin colour changes were determined with the spectrophotometer as *L**, *a** and *b**,

CIELAB values and hue was calculated using the following formula: $hue° = tan^{-1}(\frac{b*}{a*})$, (McGuire 1992) The sensing area was 7 mm^2.

The Multiplex®3 can generate a range of ultraviolet light wavelengths and can detect fluorescence signals from the plant, as well as provide plant pigment indices for anthocyanins, chlorophyll and flavonols, as described elsewhere (Ben Ghozlen et al., 2010). The evaluated fruit skin area diameter was ~1 cm. For our evaluations, the simple fluorescence ratio (SFR_R), excited with red light (635 nm), provided an estimate of the chlorophyll content, and the FLAV index provided an estimate of the flavonol content of the fruit. The indices have been described by Ben Ghozlen et al. (2010) and Betemps et al. (2012) as

$$SFR\_R = (FRF\_R/RF\_R)$$

$$FLAV = \log(FRF\_R/FRF\_UV)$$

where SFR_R is the simple fluorescence ratio, excited with red light, FLAV is the flavonol index, FRF_R is far-red fluorescence under red light excitation, RF_R is red fluorescence under red light excitation and FRF_UV is far-red fluorescence under ultraviolet excitation.

Quality analysis

To assess fruit quality, the flesh firmness, starch degradation, titratable acid (TA) and total soluble solids (TSS) content were determined. Five fruit per treatment were taken for quality determination at 0, 28, 42 and 56 days after treatment (DAT), with shelf-life ranging from 42 to 56 DAT. Fruit firmness, TSS and starch index were measured with an ART system (Apple Ripe Test; UP Products, Cottbus, Germany). Firmness was evaluated with a penetrometer (area analysed 10 mm in diameter), and fruit were tested on two opposite sides along the equatorial zone. TSS was measured with a refractometer (Pocket Refractometer PAL-1, Atago Co. Ltd, Minato, Tokyo, Japan). The starch pattern index was determined on one half of an apple after staining with an iodine solution and assessed by a black-and-white CCD camera (1=fruit flesh coloured black, no starch degradation; 10=no staining, starch free).

TA was determined by titration with 0.1 mol L^{-1} sodium hydroxide (inoLab pH Level 1; WTW, Weilheim in Oberbayern, Germany). For TA, a triple determination of a mixed sample of juice from five fruit halves per treatment was used. TA was calculated

following the Organisation for EconomicCo-operation and Development guidelines for objective tests for fruit and vegetables (OECD 2005):

$$Acid = \frac{Titre\ x\ acid\ factor\ x\ 100}{3\ (ml\ juice)}$$

where the acid factor for malic acid is 0.0067.

Experiment 4: effect of NOP-1 on ethylene and respiration of 'Golden Delicious'

Fruit, treatments and storage conditions

In this experiment, apples were harvested from the same trees as for experiment 3 and stored for 9 days at 1°C, as described earlier. Then, the same treatments as for experiment 3 were performed and fruit were then stored at 6°C for 35 days. At day 35, eight fruit per treatment were removed and used for the ethylene and respiration measurements during shelf-life.

Ethylene and respiration

Ethylene production was determined during shelf-life. For this purpose, eight fruits per treatment were divided into two replications; each replication was enclosed in an airtight glass container (4.25 L) for 2 h. Afterwards, 1 mL of headspace gas was injected into a gas chromatograph (Carlo Erba, Fractovap Series 2150, Electrometer, Mod. 180, Milano, Italy) equipped with a flame ionization detector and a stainless-steel column of 0.9 m× 1/8 in. packed with activated aluminium oxide (60 mesh). The oven temperature was set to 110°C, the injector temperature to 200°C and the temperature of the detector to 200 °C. Nitrogen was used as carrier gas (0.6 kg cm^{-2}). An external standard of 2.7 µL L^{-1} was used for calculation of the ethylene production and results are given in microlitres of ethylene per kilogram per hour.

The respiration rate was measured in CO_2 production per kilogram per hour. The measurement was done with an infrared gas analyser (URAS-2; Fa. Mannesmann, Düsseldorf, Germany) with a flow rate of 17 L h^{-1} (kg h^{-1}) using the same experimental setup as already described.

Statistical analysis

All results are expressed as means ± standard error (SE). Statistical analyses were performed by one-way analysis of variance ($\alpha \leq 0.05$) using SPSS statistic software

(PASW statistics version 25.0; SPSS Inc., Chicago, IL, USA). As post hoc analysis, Tukey's honestly significant difference (HSD; $\alpha \leq 0.05$) was used to determine differences between the treatments (NOP-1_microdispenser; NOP-1_brush, 1-MCP, control) on each measurement day. Graphs were plotted with SigmaPlot version 10 (Systat Software GmbH, Erkrath, Germany).

3. Results

Experiment 1: expression and purification of recombinant apple ethylene receptor MdETR1

Successful purification of MdETR1 was confirmed by SDS polyacrylamide gel electrophoresis and immunoblotting. Interaction of purified MdETR1 with EIN2 and stability of the EIN2– ETR1 complex was probed by MST. A dissociation constant of 136 ± 29 nmol L^{-1} (Fig. 1(A)) was obtained from these measurements, indicating a tight interaction of ETR1 and EIN2 in the complex, as observed for the corresponding tomato proteins.

Experiment 2: binding of NOP-1 to purified MdETR1

Thermophoresis was further used to monitor and to quantify the interaction of NOP-1 with purified recombinant MdETR1. The dissociation constant of the peptide–receptor complex and the related affinity of apple ETR1 for NOP-1 was calculated from changes in MST signals induced by adding different amounts of NOP-1 directly to fluorescently labelled MdETR1 (Fig. 1(B)). Clear changes of the MST signal were observed upon addition of the peptide. Selectivity of the NOP-1–receptor interaction was demonstrated in controls using chemically denatured MdETR1. Here, no changes in MST signals were observed (Fig. 1(B)). An apparent dissociation constant Kd of 14.8 ± 4.1 µmol L^{-1} was calculated from the MST experiments (Fig. 1(B)). This number compares to affinities of NOP-1 obtained with the receptor NR in tomato (Kessenbrock et al. 2017).

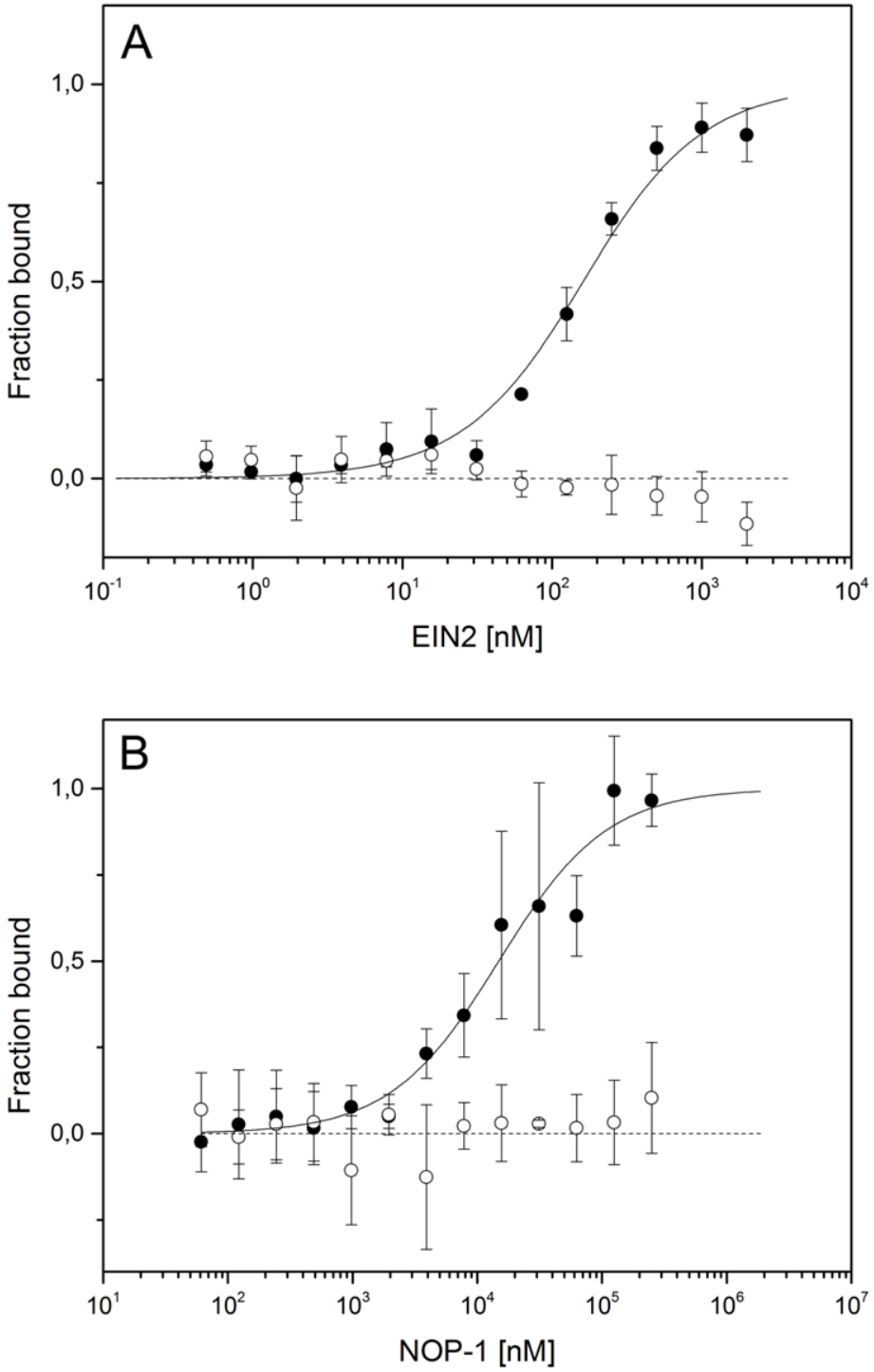

Figure 1. Interaction of apple ethylene receptor MdETR1 with EIN2 (A) studied by MST. For this interaction, a K_d value of 136 ± 29 nmol L^{-1} was calculated. (B) Interaction of MdETR1 and NOP-1 octapeptide. A K_d value of 14.8 ± 4.1 µmol L^{-1} was calculated from the corresponding MST experiment. Chemically denatured protein was used as a negative control in all experiments. No change in fluorescence was observed with the negative controls. All experiments were run in triplicate.

Experiment 3: effect of NOP-1 on fruit ripening development and quality

Sensor-based determination of fruit ripening

In our study, the colour change from green to yellow was similar in all treatments during cold storage (DAT 0–41) until DAT 25, as indicated by the hue index (Fig. 2). At DAT 32, the colour change was more advanced in the control treatment. In contrast, hue values were highest (about 100°) in 1-MCP-treated fruit, indicating green skin colour, with a significant difference at DAT 39 compared with the control. No significant difference was detected between NOP-1 treatments and between the two other treatments during cold storage. Thereafter, the fastest colour degradation during 2 weeks of shelf-life was observed in the untreated control fruit. A slower colour change over the whole shelf-life period (DAT 42–56) occurred when the NOP-1 solution was applied with a brush instead of the microdispenser. Significantly higher hue values were recorded for the 1-MCP-treated fruit.

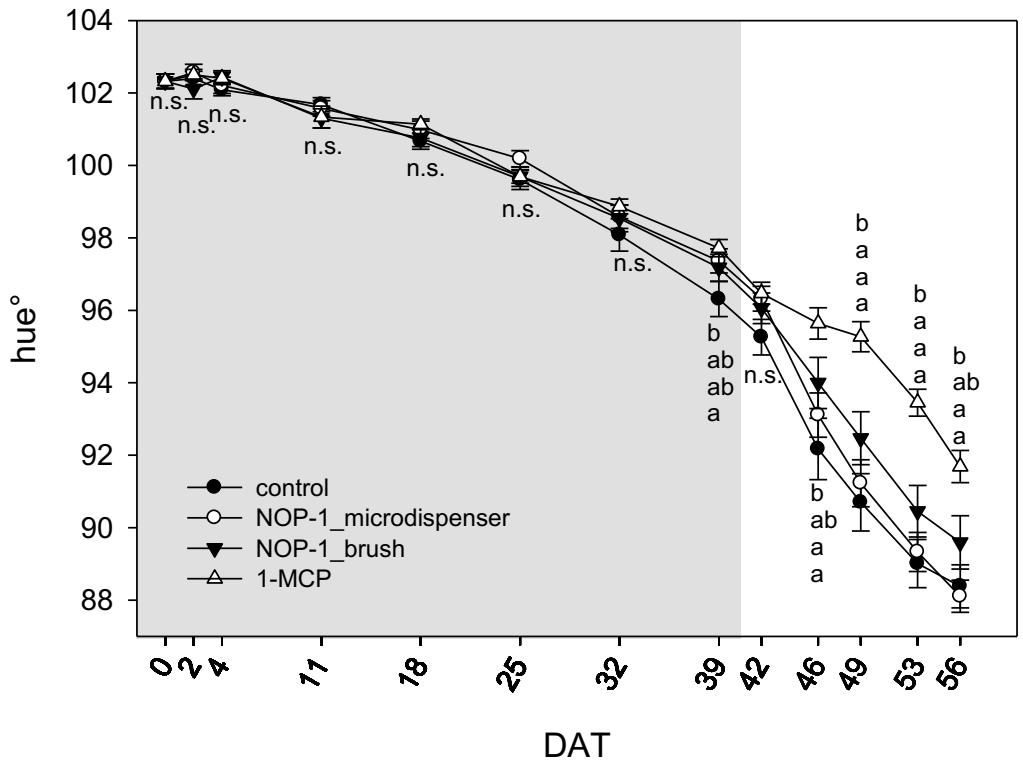

Figure 2. Hue index of apples during storage and shelf-life [0–39 DAT at cool storage (6°C) and 42–56 DAT at shelf-life (20°C)] as influenced by the experimental treatments [control fruit and fruits treated with 1000 µmol L^{-1} NOP-1 (microdispenser or brush) or with 1000 nL L^{-1} 1-MCP]. Data are means ± SE, n=15 to 5 (DAT 28, 43 and 57); different letters indicate significant differences between treatments on each measurement day (Tukey´s HSD, $\alpha \leq 0.05$).

NOP-1-treated fruit showed a slightly lower degradation of chlorophyll (SFR_R, difference 10%) during the whole shelf-life phase (DAT 42–56) compared with control fruit (Fig. 3(A)). No difference was observed between the microdispenser and brush applications. The 1-MCP treatment showed a slower decrease in SFR_R values near the end of the storage phase (DAT 39). This difference was even more pronounced during shelf-life (DAT 42–56). It is noteworthy that the NOP-1 treatment applied with a microdispenser showed a non-significant trend with a smaller decrease of SFR_R than the brush-treated applications near the end of cool storage (DAT 39) and at the beginning of the shelf-life phase (DAT 42). The FLAV index values were stable during cold storage for all treatments (Fig. 3(B)). During the shelf-life, an initial increase in FLAV indices was observed in all treatments. There were no statistical differences between all four treatments. Peak values and values at the end of shelf-life (DAT 56) were similar between the 1-MCP and NOP-1_brush application treatments, whereas NOP-1 (applied with microdispenser) showed slightly lower FLAV values (n.s.). The lowest FLAV values were recorded for control fruit towards the end of the experimental period.

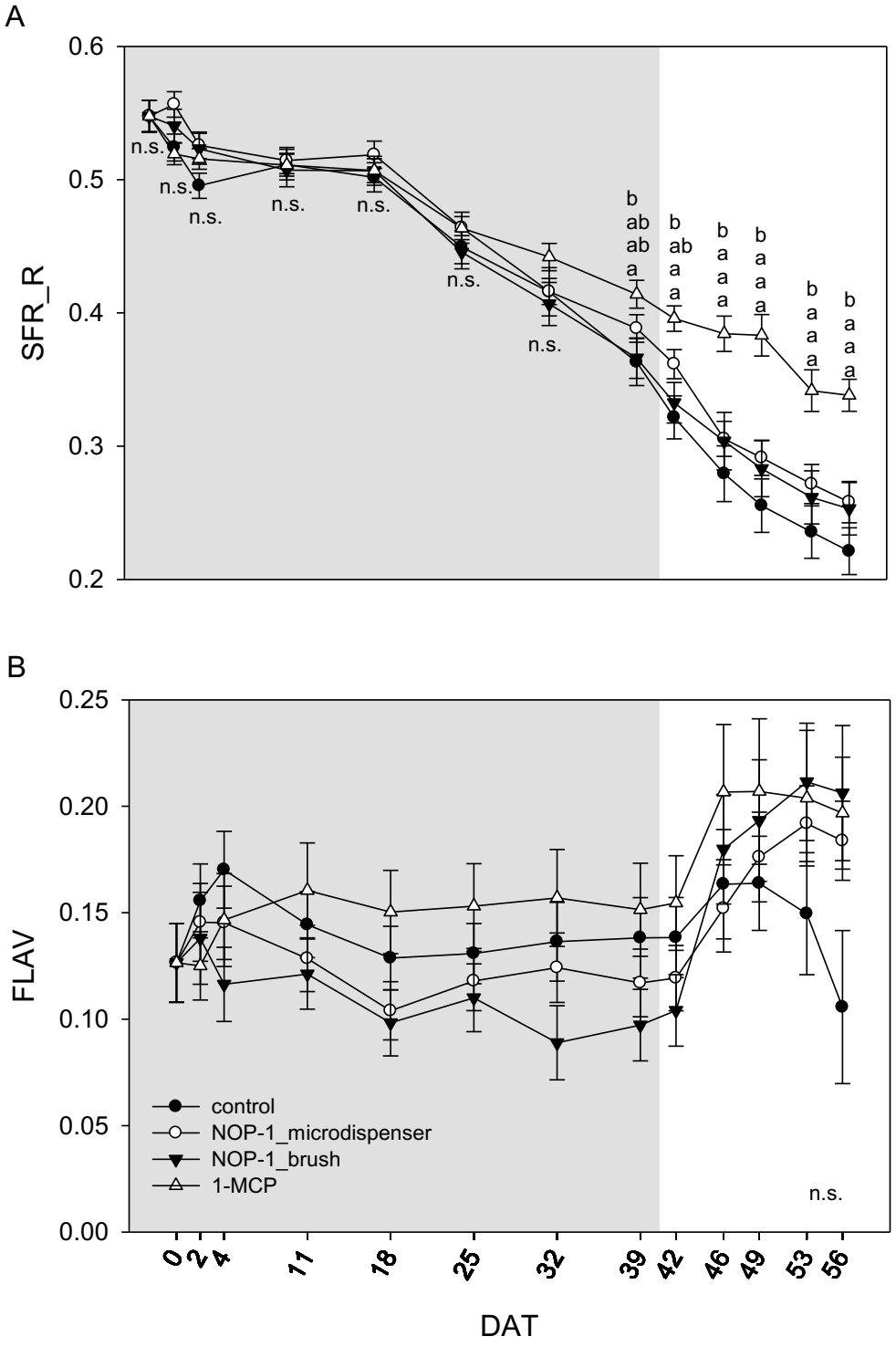

Figure 3. SFR_R index (A) and FLAV index (B) of apples during storage and shelf-life [0–39 DAT at cool storage (6°C) and 42–56 DAT at shelf-life (20°C)] as influenced by the experimental treatments [control fruit and fruit treated with 1000 µmol L^{-1} NOP-1 (microdispenser or brush) or with 1000 nL L^{-1} 1-MCP]. Data are means ± SE, n=15 to 5 (DAT 28, 43 and 57); different letters indicate significant differences between treatments on each measurement day (Tukey´s HSD, $\alpha \leq 0.05$).

Fruit quality attributes

After 4 weeks of cold storage, all treatments showed a starch breakdown of ~50% (Tab. 1). No further degradation was seen during shelf-life, and there were no significant differences between the treatments. However, TSS and TA were significantly lower in the NOP-1 treatments after 4 weeks (microdispenser) and 6 weeks (brush) of cool storage than in all other treatments. During the shelf-life, no essential changes in sugar content occurred. In contrast, a decrease in TA during shelf-life was detected in control and NOP-1_microdispenser-treated apples, whereas no further decrease in acidity was observed in the other treatments.

Fruit firmness of untreated control fruit decreased by about 20–25% during the first 4 weeks of cool storage, and about 40% by the beginning of shelf-life (42 DAT) (Tab. 1). The decrease in fruit firmness in the 1-MCP treatment was only half of this, with 10% and 20% after 4 weeks and 6 weeks of cold storage respectively. Compared with untreated control fruit, the decrease in firmness in the NOP-1 treatments was lower (about 6% DAT 28), but not significantly different. During the shelf-life, there were no further changes in flesh firmness in any treatment group. At the end of shelf-life (56 DAT), 1-MCP-treated apples still showed higher fruit firmness than all other treatments.

Table 1. Development of quality attributes of 'Golden Delicious' apples as influenced by the experimental treatments after cool storage and shelf-life simulation [DAT 28 at cool storage (6°C), DAT 42 and DAT 56 at shelf-life (20°C)] for control fruit and fruit treated with NOP-1 (microdispenser or brush) and 1000 nL L^{-1} 1-MCP. Data are means ± SE, n=5; different letters indicate significant differences between treatments on each measurement day (Tukey´s HSD, $\alpha \leq 0.05$). The bold numbers in Tab.1 identify Day after Treatment (DAT).

Treatment	**Flesh firmness (N)**		**Starch index (1 to 10)**		**TSS (Brix°)**		**TA (%)**	
Initial evaluation [DAT 0]	71.6 ± 0.3		6.3 ± 0.6		11.5 ± 0.3		0.6 ± 0.1	
Cold storage (6°C) [DAT]	**28**		**28**		**28**		**28**	
Control	52.0 ± 0.1^{a}		9.0 ± 0.1^{a}		12.5 ± 0.5^{a}		0.6 ± 0.0^{a}	
NOP-1_microdispenser	54.2 ± 0.2^{a}		9.5 ± 0.1^{b}		10.0 ± 1.8^{a}		0.6 ± 0.0^{a}	
NOP-1_brush	56.3 ± 0.1^{a}		9.3 ± 0.1^{ab}		12.9 ± 0.2^{a}		0.5 ± 0.0^{b}	
1-MCP	63.9 ± 0.1^{b}		9.3 ± 0.1^{a}		12.2 ± 0.2^{a}		0.6 ± 0.0^{a}	
Shelf-life (20°C) [DAT]	**42**	**56**	**42**	**56**	**42**	**56**	**42**	**56**
Control	43.7 ± 0.2^{a}	43.2 ± 0.2^{a}	9.3 ± 0.1^{ab}	9.5 ± 0.1^{a}	12.7 ± 0.4^{a}	12.3 ± 0.6^{a}	0.6 ± 0.01^{a}	0.4 ± 0.0^{a}
NOP-1_microdispenser	44.9 ± 0.2^{a}	44.9 ± 0.2^{a}	9.5 ± 0.0^{b}	9.6 ± 0.1^{a}	12.3 ± 0.2^{a}	12.2 ± 0.3^{a}	0.6 ± 0.00^{a}	0.4 ± 0.0^{a}
NOP-1_brush	43.6 ± 0.3^{a}	42.1 ± 0.1^{a}	9.3 ± 0.0^{a}	9.6 ± 0.0^{a}	12.7 ± 0.0^{a}	11.5 ±0.4^{a}	0.5 ± 0.1^{b}	0.4 ± 0.0^{a}
1-MCP	59.6 ± 0.2^{b}	62.6 ± 0.5^{b}	9.4 ± 0.1^{ab}	9.6 ± 0.1^{a}	13.2 ± 0.1^{a}	12.5 ± 0.5^{a}	0.6 ± 0.0^{a}	0.5 ± 0.0^{b}

Experiment 4: effects of NOP-1 on ethylene production and respiration

The highest ethylene release was observed for the NOP-1 treatments and untreated control. Somewhat surprisingly, during the first week of shelf-life, the NOP-1 treatments showed the highest ethylene production rate. Concerning the different application techniques of NOP-1 (microdispenser and brush), microdispenser showed higher ethylene production than the brush application did. There was no detectable ethylene production during shelf-life for 1-MCP-treated fruit (Fig. 4(A)).

The respiration rate was highest in fruit treated with NOP-1, even though there was no difference concerning the application technique (Fig. 4(B)). The lowest CO_2 release rate was recorded in the 1-MCP treatments, which showed 70% less respiration than the NOP-1 treatments, and 60% less than the untreated control.

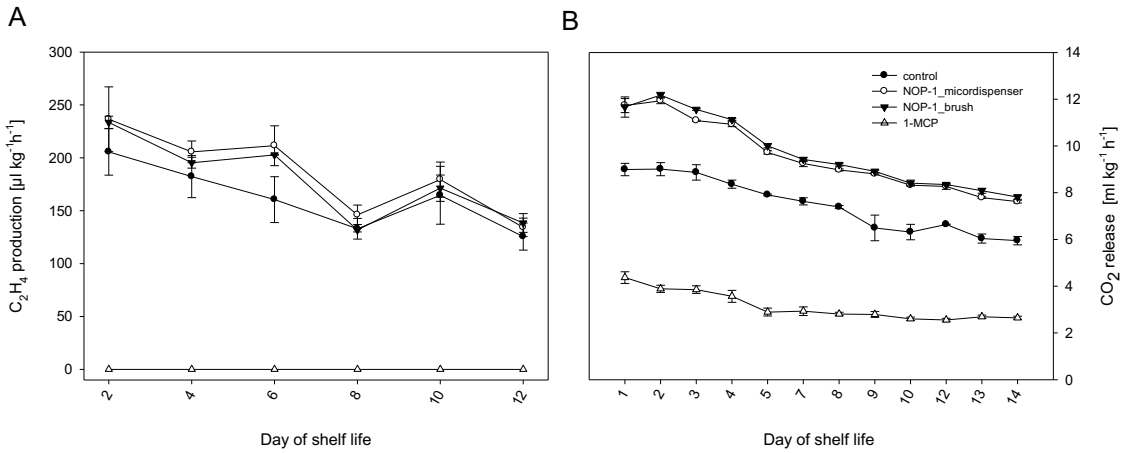

Figure 4. Ethylene production (A) and respiration (B) during shelf-life (20°C) of control apples and fruit treated with 1000 µmol L^{-1} NOP-1 (microdispenser or brush) or with 1000 nL L^{-1} 1-MCP. Data are means ± SE, n=2 × 4 fruit.

4. Discussion

Recent studies have confirmed the effectiveness of the NLS-derived octapeptide NOP-1 to delay tomato ripening (Bisson et al., 2016; Kessenbrock et al., 2017). Owing to the homology of the ethylene receptors and the ubiquitous presence of the NLS motif in the plant kingdom, it is likely that the ripening inhibition effects of NOP-1 can also apply to other climacteric fruits (Bisson et al., 2016; Kessenbrock et al., 2017). Encouraged by our binding studies of NOP-1 to purified recombinant MdETR1 that indicate binding of the peptide to the apple ethylene receptor at similar affinities as for the related tomato proteins, we investigated for the first time the efficacy of NOP-1 on apple. Experiments with surface applications of NOP-1 were conducted on ‘Golden Delicious’, as it is one of the most important apple cultivars worldwide. In addition, different application techniques of NOP-1 were compared with untreated and 1-MCP-treated fruits.

Our results show a minor extension of shelf-life by NOP-1 with delays in colour development (Fig. 2) and chlorophyll degradation (Fig. 3(A)). According to the results obtained in tomatoes, NOP-1 mainly seems to delay ripening-related changes in pigment concentrations (Kessenbrock et al., 2017). In order to test potential practical applications, NOP-1 was applied with a microdispenser to mimic a spray solution. However, only a minimal inhibition of ripening was seen in the hue values (Fig. 2). In contrast, the SFR_R index, an estimate of chlorophyll concentration (Fig. 3(A)), indicated a similar ripening delay for fruit treated with either a brush or microdispenser. Sprayable formulations of pre-harvest ripening inhibitors like aminoethoxyvinylglycine are very valuable to optimize harvest management and fruit quality, as fruit can stay on trees for a longer

period, thereby extending the harvest window and allowing an increase in certain quality attributes such as size and colour (Byers and Eno, 2002).

The results presented here indicate that NOP-1 applied with a brush (as surface film) seems to be more effective to delay apple ripening than when applied with a microdispenser (Fig. 2). Bisson et al., (2016) also showed that surface applications with a brush were more efficient than injected or incubated treatments. One reason for the higher efficacy of brush applications could be changes in the epicuticular wax microstructure. Wax reduces the permeability of water-soluble substances (Kolattukudy, 1984). Fruit wax functions as a barrier with high impermeability for the movement of compounds in any direction (Kolattukudy, 1984). When NOP-1 is applied by brush, the permeability of the wax barrier may have been altered or decreased, so that NOP-1 was able to better penetrate through the cuticle. The entirely different composition of their cuticule (Martin and Rose, 2014) and the related compositional diversity in cuticular waxes (Szakiel et al., 2012) may also account for the reduced effect of NOP-1 on ripening in apples compared with tomatoes.

1-MCP significantly delayed all ripening parameters and strongly suppressed ethylene production and respiration activity (Figs 2–4). In contrast, NOP-1 did not have any inhibiting effect on ethylene synthesis or respiration during shelf-life. Its application modes might induce a stress reaction with consecutive elevation of ethylene and CO_2 production (Fig. 4) (Bisson et al., 2016). These results are in accordance with a previous investigation of NOP-1 on tomato ripening without any impairment of ethylene biosynthesis (Bisson et al., 2016). This might be due to the different target points of 1-MCP and NOP-1 in the ethylene signalling pathway. Whereas 1-MCP blocks ETR, NOP-1 causes downstream impairment through an ETR–EIN2 interaction (Bisson and Groth, 2015). However, NOP-1 appears not to affect autocatalytic ethylene production or respiration and this might be one reason to explain its weak influence on the ripening of ‘Golden Delicious’ apples (Figs 2, 3(A), and 4) (Blankenship and Dole, 2003).

Moreover, the weak effect on apple fruit may also be related to the lower affinity of the NOP-1 peptide to the apple ethylene receptor. Although the binding affinity determined in our studies with the purified recombinant MdETR1 receptor compares with numbers previously detected with tomato NR receptor (K_d =23.5 μmol L^{-1}), tomato receptors LeETR1 (K_d =0.2 μmol L^{-1}) and LeETR4 (Kd =4.1 μmol L^{-1}) showed much stronger binding affinity of NOP-1 in the sub and lower micromoles per litre range. Application of higher concentrations of NOP-1 in future studies will reveal whether stronger effects

of the peptide biological on apple ripening might be reached.

In addition, the differences in ethylene production and ethylene sensitivity between apple and tomato are important to take into account (Kessenbrock et al., 2017; Martínez-Romero et al., 2007). In this case, the higher ethylene production rate and higher ethylene sensitivity in apple could be another reason to explain the weaker delaying effect of NOP-1 (Kessenbrock et al., 2017; Martínez-Romero et al., 2007). NOP-1 penetration through the cuticle and uptake into the cells seems to be another critical factor to achieve a delay in ripening (Bisson et al., 2016). NOP-1 might be taken up by oligopeptide transporters, which are responsible for peptide transport (Bisson et al., 2016). A direct uptake on the basis of the inherent molecular properties (short; water solubility; partly hydrophobic and/or polybasic structure; positive net charge of physiological pH) is also possible (Bisson et al., 2016; Järver and Langel, 2006). However, the penetration rate of NOP-1 through the apple cuticle is as yet unclear.

The fruit quality parameters showed no differences between NOP-1-treated apples and control fruit (Tab. 1). This includes ethylene-dependent maturity parameters like firmness and chlorophyll degradation, as well as ethylene-independent factors such as sugar accumulation and decrease of acidity (Tab. 1, Fig. 3(A)) (Pech et al., 2008). Interestingly, ethylene-independent quality attributes were not impaired by 1-MCP. However, firmness was markedly maintained in the 1-MCP treatments, indicating inhibition of cell-wall-modifying enzymes, which apparently did not occur in NOP-1-treated fruits (Tab. 1). 1-MCP irreversibly blocks ethylene receptors with consecutive inhibition of all ethylene-dependent reactions (Blankenship and Dole, 2003). NOP-1 is a competitive inhibitor of the EIN2–ETR1 interaction, which does not impair ethylene autocatalysis (Bisson et al., 2016). It is assumed that the reversible inhibition of the EIN2–ETR1 interaction does not affect all ethylene response functions, like ethylene-dependent changes in fruit firmness (pectins and xyloglucans) (Pech et al., 2008). Focusing on the competitive inhibition mechanism of NOP-1 at the kinase domain of the ETR, repetitive application might be more effective to better understand how NOP-1 affects ripening (Bisson and Groth, 2011). With regard to flavonols, both NOP-1 and 1-MCP treatments led to higher concentrations than the control treatment (Fig. 3(B)) (Betemps et al., 2012), suggesting that, even though ethylene is involved in the regulation of the flavonoid biosynthesis, NOP-1 and 1-MCP do not adversely affect flavonol content in this cultivar.

5. Conclusion

Our study showed that EIN2 tightly interacts with the MdETR1, and the NLS-derived octapeptide NOP-1 also had binding activity to the receptor. In contrast to studies with tomato, NOP-1 only slightly delayed 'Golden Delicious' apple ripening, whereas there were no negative effects on fruit quality parameters. The delaying effect of NOP-1 was more pronounced when the peptide was applied by a brush than by a microdispenser, which might be explained by physical effects to the wax layer. In contrast to gaseous 1-MCP, which strongly inhibited ripening by suppressing ethylene and CO_2 production, NOP-1 did not show an influence on either ethylene release or respiration rates. The weak effect of NOP-1 on apple compared with tomato might be caused by the differences in the amount of ethylene production and/or differences in ethylene sensitivity and the lower affinity of NOP-1 to MdETR1. Overall, NOP-1 might offer a novel way of ripening control of climacteric fruit. Nevertheless, it is necessary to better understand the action of the peptide in different species. Moreover, further studies are needed to enhance the efficacy of NOP-1; for example, by examining the penetration of NOP-1.

6. Acknowledgements

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D Ripening inhibitory effect of the ethylene signalling protein EIN2 derived octapeptide NOP-1 on tomatoes as influenced by repeated applications and applications at different ripening stages[3]

1. Introduction

Ripening of climacteric fruits is mainly regulated by the plant hormone ethylene, a short-chain alkene (Brady, 1987; Lelievre et al., 1997; Wang et al., 2002). Fruit ripening is accompanied by physiological, morphological and chemical changes within the fruit leading to flesh softening, colouring and aromatisation (Batu, 2004; Brady, 1987). To improve shelf-life, reduce food spoilage and maintain quality of tomato fruits, different methods might be used to reduce the synthesis and release of ethylene, or the consequences of its action at enzymatic and cellular levels (Kessenbrock et al., 2017). Amongst others, manipulation of storage conditions, enzyme inhibition (e.g. aminoethoxyvinylglycine, nitric oxide), genetic engineering and application of ethylene antagonists like 1-Methylcyclopropene (1-MCP) (Eum et al., 2009; Kessenbrock et al., 2017; Saltveit, 2005;) might be adopted. The gaseous treatment with 1-MCP at the beginning of storage, is a well-established technique for inhibiting ripening and extending shelf-life and quality of climacteric fruits (Blankenship and Dole, 2003; Poyesh et al., 2018). 1-MCP is a receptor-antagonist of ethylene and strongly suppresses maturation and autocatalysis of ethylene (Poyesh et al., 2018). New insights into the ethylene signalling pathway have led to the successful testing of a peptide-based product to delay ripening of climacteric fruits (Bisson et al., 2016). The nuclear localization signal octapeptide1 (NOP-1), a short peptide derived from the C-terminal end of the ethylene regulator protein ethylene-insensitive-2 (EIN2), prevents the EIN2-ETR1 (ethylene receptor 1) complex formation which is necessary for the downstream ethylene signalling (Bisson et al., 2016). As shown, NOP-1 delayed ripening of tomatoes when applied in early maturity stages without impairing ethylene formation nor having negative impact on fruit quality (Bisson et al., 2016). Although the ripening delay of

[3]Klein, S., Fiebig, A., Noga, G., Groth, G., Hunsche, M., (2019): Ripening inhibitory effect of the ethylene signalling protein EIN2 derived octapeptide NOP-1 on tomatoes as influenced by repeated applications and applications at different ripening stages. In: *Scientia Horticulturae* 254, S. 143–147.

NOP-1 is weaker compared to 1-MCP, the preservation of fruit quality is favorable. 1-MCP, when applied in an early maturity stage (mature green; MG), has shown negative effects on specific quality attributes such as firmness and aroma (Hurr et al., 2005; Mir et al., 2004). Interestingly, the application of 1-MCP in more advanced maturity stages of tomatoes such as pink (PI) and light red might also extended the ripening process, similar to repeated application (Mir et al., 2004). Contrasting to this, the effect of an exposure to NOP-1 on the ripening behavior of tomatoes in the stages breakers (BR) and pink, or even the effect of sequential applications, have not yet been studied. Therefore, we evaluated the influence of the surface application of NOP-1 on the ripening development tomatoes after repeated administration and when applied in the maturity stages BR and PI.

2. Material and methods

Experiment 1: ripening-delay through repeated application of NOP-1

Experimental design

Tomato fruits (*Solanum lycopersicum L.*) of the cultivar 'Lyterno', which were cultivated in a commercial-like greenhouse at the research station Campus Klein-Altendorf (University of Bonn, Germany), were harvested at the maturity stage "green" (USDA, 1991). For the experiment, the last two tomato fruits at the distal end of the fourth truss were harvested, whereby the trusses had previously been thinned to six fruits per truss. For the experiment, 16 fruits per treatment of identical size and maturity stage were harvested, cleaned with water and randomized. For the first measurement before treatment application, all selected fruits were evaluated non-destructively and then randomly allocated to different treatments, as follows:

control (untreated);

NOP-1, 1000 µM: applied on DAT 0;

NOP-1, 1000 µM_repeated: applied on DAT 0 and DAT 7;

1-MCP: 1000 nL L^{-1}

NOP-1 peptides (GenScript, Piscataway, NJ, United States) were dissolved in a FRET-buffer modified after Bisson et al. (2016) (50 mM Tris−HCl pH 8.0, 300 mM NaCl, 0.015% (w/v) Fos-Choline-16, 0.05% (v/v) Tween 20). On each fruit, a total of 200 microdroplets (0.5 µL each) were applied with a Hamilton microdispenser (Hamilton

Bonduz AG, Bonduz, Switzerland) over the whole tomato surface. Based on previous dose-finding studies on tomatoes, a NOP-1 concentration of 1000 µM was used (Kessenbrock et al., 2017). A concentration of 1000 nL L^{-1} (1.6 g/m3 of SmartFreshSM 0.14% active substance) was used for the 1-MCP treatment (SmartFresh, Agrofresh Milano, Italy), according to the standard recommendations of the manufacturer. Untreated fruits served as control treatment. After application of the ripening inhibitory substances, each treatment was allocated in a storage box and kept at room temperature (19 ± 2°C) for 21 days.

Non-destructive evaluations of fruit ripening

For the non-destructive monitoring of fruit ripening development during the trial period (DAT 0–21), one sensor based on light reflection (Minolta) and one based on fluorescence signals (Multiplex®) were used. For the evaluations, three different points at the fruit equatorial zone were selected. The portable spectrophotometer with a sensing area of 7 mm^2 (CM-700d, Konica Minolta Inc., Tokyo, Japan), based on the CIELAB model, is able to determine changes in fruit surface colour through the hue° index. Hue° was calculated according to the method described by McGuire (1992). The fluorescence-based equipment, a hand-held multiparametric sensor (Multiplex®3, Force-A, Orsay, France) equipped with light-emitting diodes with excitation channels at UV (375 nm), blue (475 nm), green (510 nm) and red (635 nm) spectral zones, was used. Fluorescence signals were detected in the spectral regions of blue-green (BGF, 425–475 nm), red (RF, 680–690 nm) and near-infrared (FRF, 720–755 nm) (Ben Gholzen et al., 2010). Based on the absolute fluorescence signals detected in a diameter of approximately 2 cm, fluorescence ratios were calculated. In this experiment, the Multiplex index SFR_R (Simple Fluorescence excited with red light) was used to estimate changes in the chlorophyll content during the ripening process, as described by. Ben Ghozlen et al. (2010).

Destructive determinations of fruit quality

Flesh firmness

For detection of cell-wall polymers breakdown, the flesh firmness of each 5 tomato fruits was measured on the equatorial zone at the beginning (DAT 0; DAT 4) and at the end (DAT 21) of the experiment using a hand-held penetrometer (Härteprüfer DIN 53 505, Bareiss, Oberdischingen, Deutschland) with a diameter of 3 mm. The measuring results were converted from Shore (Sh_A) to Newton (N), using the following formula (Kunz and Studer, 2006)

$$F = C_1 + C_2 \times Sh_A[N]$$

$$C_1 = 0.549\ N, C_2 = 0.07516\ N$$

Determination of chlorophyll, ß-carotene and lycopene.

Chlorophyll, ß-carotene and lycopene content were determined photometrically according to the method of Nagata and Yamashita (1992) as described by Barros et al. (2007); Azeez et al., (2012) and Kalogeropoulos et al. (2012). Briefly, 0.1 g of freeze-dried and ground tomato samples were solved in acetone: hexane (4:6), homogenized and centrifuged for 10 min at 16,100 x g (CENTRIFUGE 5415 R, Eppendorf AG, Hamburg, Germany). The resulting supernatant was filled with the solvent to 2 ml and the absorption of this solution was determined at 453, 505, 645 and 663 nm in a spectrophotometer (LAMBDA 35, PerkinElmer®, Waltham, MA, United States). The chlorophyll *a*, chlorophyll *b* (total chlorophyll content: Chl a+ Chl b), ß-carotene and lycopene concentrations were calculated according to the method of Nagata and Yamashita (1992)

Experiment 2: ripening delay through NOP-1 application at advanced maturity stages

In this experiment, fruits of the same cultivar as in experiment 1 were harvested at the stages BR and PI (USDA, 1991). Fruits were then assorted to the following treatments (n=21):

- control_BR;
- NOP-1_1000µM_BR;

- control_PI;
- NOP-1_1000µM_PI.

NOP-1 peptides were dissolved in a buffer as described above and applied with a microdispenser (4x50 microdroplets of 0.5 µL volume on each fruit) while the control fruits remained untreated. The experimental period lasted for 16 days. Storage conditions, evaluation devices, measurement methods and analytical procedures were the same as described above. For the destructive analysis 5 tomato fruits per treatment were used at the start (DAT 0; DAT 4) and at the end (DAT 16) of the experiment.

Classification of ripening stages of tomato fruits

The fruit ripening stage of tomato was selected based on the *Standards for Grade of Fresh Tomatoes* (USDA, 1991). It is divided into 6 maturity stages, which describe the colour stages of tomato during ripening. The mature stages are named after the colour of the tomato: Stage 1 = green (mature green), Stage 2 = colour break from green to red (breakers), Stage 3 = turning (10–30% of surface redish), Stage 4 = pink (30–60 % of surface redish), Stage 5 = light red (> 60% of surface redish), Stage 6 = red (100% red surface).

In this experiment tomatoes of the ripening stages green (mature green); breakers and pink were used.

Statistical analysis

All results are expressed as means ± SE. For experiment 1, means of data were compared by Analysis of Variance (one-way ANOVA ($\alpha \leq 0.05$)). In case of statistical significance, the Tukey´s HSD ($\alpha \leq 0.05$) was applied to establish differences. A t-test was used to analyse results obtained from experiment 2. Statistical analysis was performed with the programme IBM® SPSS® Statistics version 22 (IBM Corporation, USA), and graphs were prepared with SigmaPlot version 10 (Systat Software GmbH, Erkrath, Germany).

3. Results

Experiment 1: ripening-delay through repeated application of NOP-1

The colour change from green to red, as indicated by the hue° index, was significantly delayed from DAT 12 up to DAT 17 in fruits treated with NOP-1 as compared to the control fruits (Fig. 1A). After DAT 17, hue° index of NOP-1 treated fruits was similar to the control treatment.

No difference between the treatments ‘single application’ (DAT 0) and ‘repeated application’ (DAT 0 and DAT 7), was noted (Fig. 1A). The application of NOP-1 also resulted in significantly higher SFR_R values, without having an additional effect on chlorophyll breakdown through repeated NOP-1 application (Fig.1B). Surprisingly, unlike the hue° index, SFR_R index of NOP-1 treated fruits showed an ongoing ripening delay effect even on DAT 21 (Fig. 1B). The strongest ripening delay was observed when using 1-MCP. This was confirmed by evaluation of fruit maturity characteristics with both reflection and fluorescence systems (Fig. 1A, DAT 9–21, and 1B, DAT 10–21).

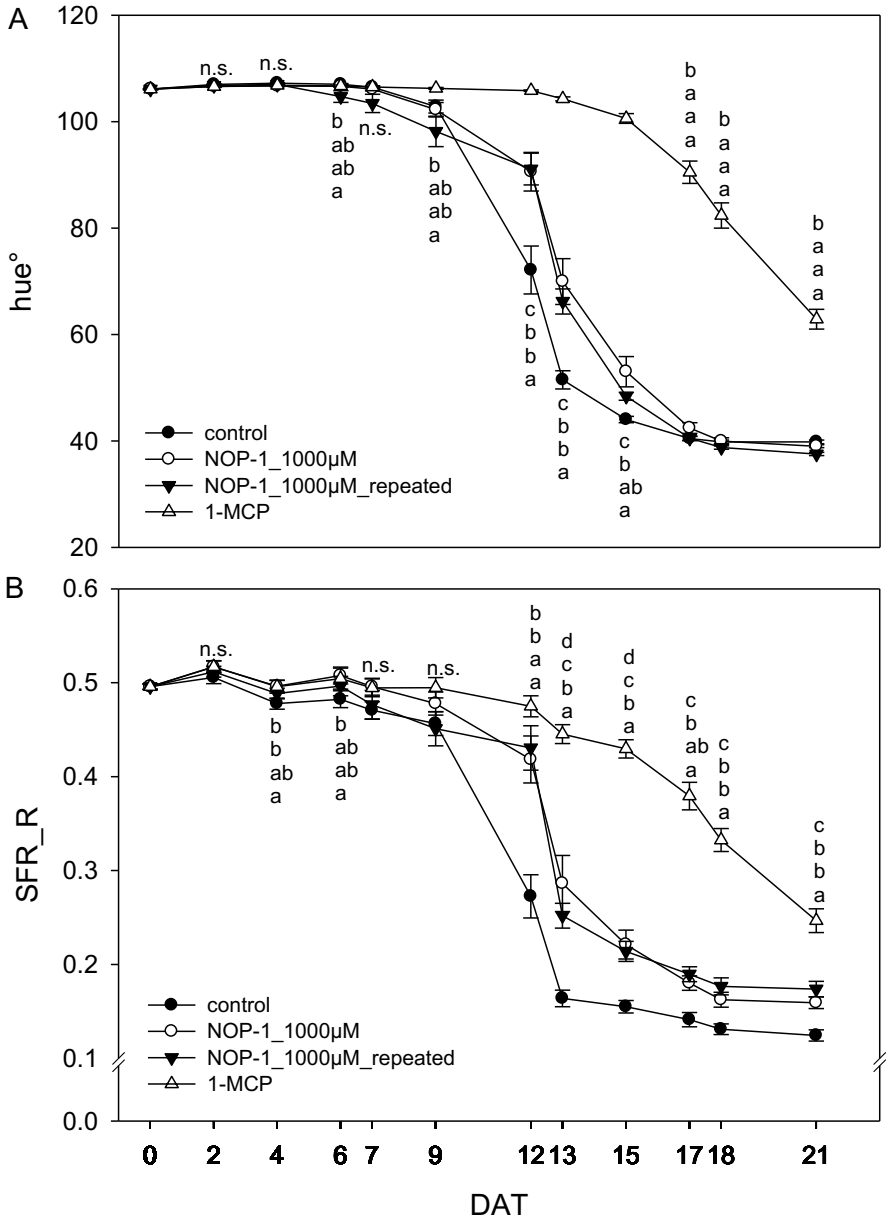

Figure 1. Hue° index (A) and SFR_R index (B) of tomato fruits at MG (mature green) as influenced by the experimental treatments (control fruits and fruits treated with 1000µM NOP-1[single (DAT 0) or repeated (DAT 0 and 7) application] or with 1000 nL L^{-1} 1-MCP. Data are means ± SE, n=3×16, – 5 on DAT 4; 21, different letters indicate significant differences between treatments on each measurement day (Tukey's HSD, $\alpha \leq 0.05$).

Flesh firmness of NOP-1 treated green tomatoes and untreated green tomatoes decreased during the experiment from 6.6 – 6.5 N to 4.4 – 4.3 N (Tab. 1). However, no significant differences between control and NOP-1 treated fruits were detected. Repeated NOP-1 application did not show any additional effect on delaying or avoiding flesh firmness decrease. Softening of flesh firmness in 1-MCP treated fruits was significantly delayed when compared to the other treatments, ending with 5.4 N at DAT 21. Nevertheless, decrease in cell wall firmness over time was observed in all treatments. Finally, there

were no differences in the contents of chlorophyll, lycopene and ß-carotene between control fruits and NOP-1 treated fruits (Tab. 1). 1-MCP significantly delayed chlorophyll breakdown (DAT 21), whereas changes in lycopene and ß-carotene content were similar to control and NOP-1 treated fruits (Tab. 1).

Table 1. Development of quality attributes of mature green tomatoes as influenced by the experimental treatments (at DAT 4 and DAT 21) for control fruit and fruits treated with 1000 µM NOP-1 (single (DAT 0) or repeated (DAT 0 and 7) application) or with 1000 nL L−1 1-MCP). Data are means ± SE, n=5, different letters indicate significant differences between treatments on each measurement day (Tukey´s HSD, $\alpha \leq 0.05$).

Treatment	Flesh firmness [N]		Chlorophyll [mg/g] (*e^{-2})		Lycopene [mg/g] (*e^{-2})		ß-carotene [mg/g] (*e^{-2})	
starting point DAT 0	7.2 ± 0.6		11.7 ± 1.2		1.1 ± 0.3		3.5 ± 0.1	
DAT	**4**	**21**	**4**	**21**	**4**	**21**	**4**	**21**
Control	6.6 ± 0.6	4.3 ± 0.7^{a}	10.7 ± 1.1	0.5 ± 0.2^{a}	1.5 ± 0.2	1.8 ± 0.1	3.2 ± 0.2^{ab}	4.4 ± 0.2
NOP-1_1000µM_1	6.5 ± 0.65	4.5 ± 0.74^{a}	11.3 ± 0.9	0.9 ± 048^{a}	1.6 ± 0.2	2.3 ± 0.2	3.3 ± 0.0^{ab}	5.2 ± 0.3
NOP-1_1000µM_1-7	6.6 ± 0.63	4.2 ± 0.67^{a}	11.3 ± 0.6	1.3 ± 0.2^{a}	1.7 ± 0.2	2.2 ± 0.2	3.1 ± 0.1^{ab}	5.0 ± 0.5
1-MCP	6.8 ± 0.59	5.4 ± 0.66^{b}	11.8 ± 0.4	3.0 ± 0.7^{b}	2.3 ± 0.4	2.1 ± 0.2	2.6 ± 0.2^{a}	5.3 ± 0.4

Experiment 2: ripening delay NOP-1 application at advanced maturity stages

Application of NOP-1 at the maturity stage BR led to a significant ripening delay as proven by hue° index and SFR_R index (Fig. 2A and C). Colour change (hue° index) from maturity stage BR to red was slowed down by NOP-1 for about seven days (DAT 2–7) (Fig. 2A). Chlorophyll breakdown was inhibited for a longer period by application of NOP-1, leading to a significant difference in SFR_R until the end of the experiment (DAT 21) in comparison to untreated fruits (Fig. 2C). The application of NOP-1 on tomatoes of the maturity stage PI showed no detectable effect on colour change, as both hue° and SFR_R did not significantly differ between the treatments (Fig. 2B and D). Fruit firmness decreased by about 50% in BR fruits from DAT 0 to DAT 16, independently of NOP-1 application (Tab. 2). In PI fruits firmness breakdown was similar to BR with slightly higher firmness of the treated fruits at DAT 16 (Tab. 2). NOP-1 applied at ripening stage BR delayed chlorophyll breakdown (4 DAT: 2.2 vs. 1.6 mg/g);

16 DAT: 0.5 vs. 1.0 mg/g; Tab. 2) without reaching statistical significance. The increase of lycopene content during maturation was delayed by NOP-1 (16 DAT; Tab. 2). At DAT 16 lycopene content of NOP-1 treated fruits was significantly lower in both BR and PI (Tab. 2). No significant differences in chlorophyll and ß-carotene concentrations between control and NOP-1 treated fruits were detected.

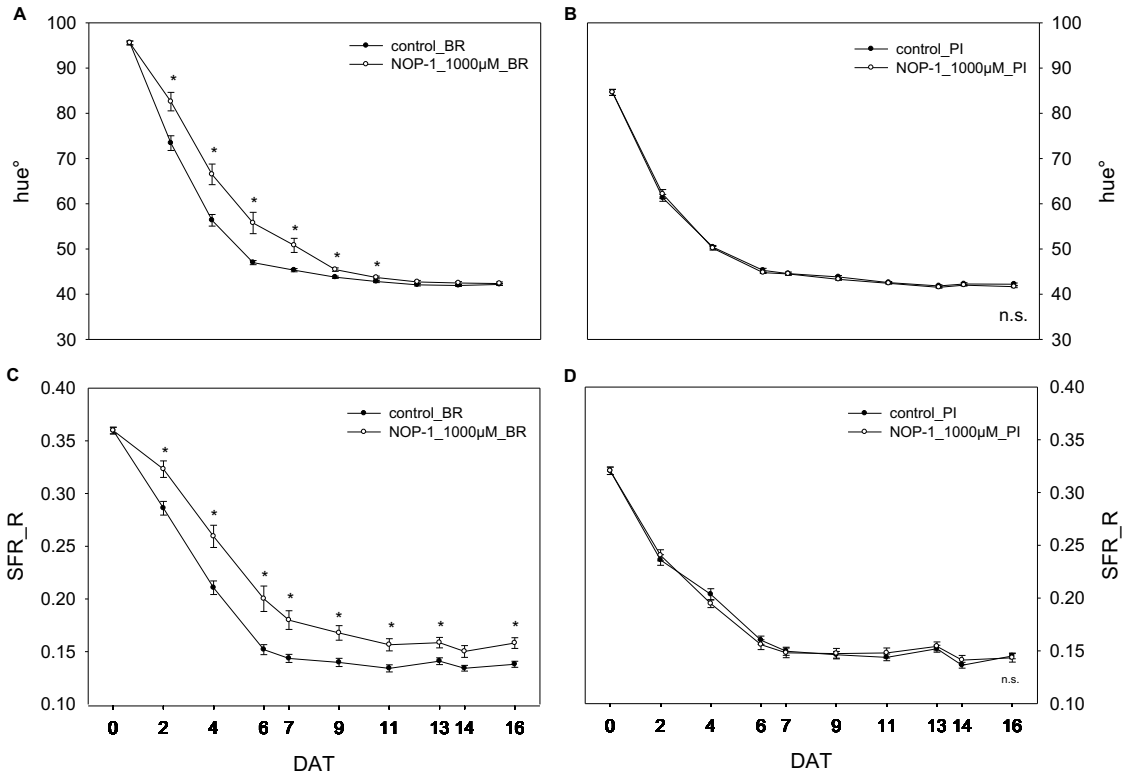

Figure 2. Hue° index (A; B) and SFR_R index (C;D) of tomatoes as influenced by NOP-1treatment at different maturity stages (control fruit at maturity stages BR (breakers) and PI (pink), and fruits at maturity stages BR and PI treated with 1000 µM NOP-1). Data are means ± SE, n=3×21, – 5 on DAT 4; 16, significant differences ($P \leq 0.05$) between the treatments: control_BR and NOP-1_1000µM_BR; control_PI and NOP-1000µM_PI for each measurement day assessed by *t*-test.

Table 2. Development of quality attributes of tomatoes as influenced by NOP-1treatment at different maturity stages (at DAT 0, 4 and DAT 2) for control fruit at maturity stages BR (breakers) and PI (pink), and fruits at maturity stages BR and PI treated with 1000 µM NOP-1. Data are means ± SE, n=5, significant differences ($P \leq 0.05$) between the treatments: control_BR and NOP-1_1000µM_BR; control_PI and NOP-1000µM_PI for each measurement day assessed by *t*-test.

Treatment	**Flesh firmness [N]**		**Chlorophyll [mg/g] (*e^{-2})**		**Lycopene [mg/g] (*e^{-2})**		**ß-carotene [mg/g] (*e^{-2})**	
starting point BR DAT 0	6.9 ± 0.65		5.1 ± 0.2		0.8 ± 0.2		5.1 ± 0.4	
DAT	**4**	**16**	**4**	**16**	**4**	**16**	**4**	**16**
control_BR	5.7 ± 0.62	3.7 ± 0.63	1.6 ± 0.3	0.5 ± 0.1	6.1 ± 0.6	9.3 ± 0.2*	7.6 ± 0.1	8.2 ± 0.1
NOP-1_1000µM_BR	5.6 ± 0.71	3.8 ± 0.72	2.2 ± 0.5	1.0 ± 0.3	5.1 ± 0.8	8.2 ± 0.2	7.3 ± 0.3	8.2 ± 0.2
Starting point PI DAT 0	6.7 ± 0.64		4.6 ± 0.4		1.6 ± 0.2		5.7 0.4	
DAT	**4**	**16**	**4**	**16**	**4**	**16**	**4**	**16**
Control_PI	5.5 ± 0.65	3.4 ± 0.62	1.1 ± 0.1	0.7 ± 0.1	7.8 ± 0.7	8.4 ± 0.5*	7.8 ± 0.3	8.5 ± 0.2
NOP-1_1000µM_PI	5.4 ± 0.68	3.7 ± 0.65*	1.1 ± 0.1	0.5 ± 01	8.0 ± 0.5	6.6 ± 0.3	7.4 ± 0.1	8.5 ± 0.2

4. Discussion and conclusion

Impairment of the ethylene signalling pathway by application of the octapeptide NOP-1, which is derived from the NLS (Nuclear localisation signal) motive of the ethylene regulator protein EIN2, delays ripening of tomatoes when applied during the early ripening stage (stage mature green) (Bisson et al., 2016; Kessenbrock et al., 2017). This result was confirmed in this study, even though the ripening inhibitory effect of NOP-1 was weaker when compared to 1-MCP, evidenced by hue° values (Fig. 1 A and SFR_R index, Fig. 1 B) and chlorophyll content (Tab. 1). The influence of ripening parameters by repeated NOP-1 applications and applications at different ripening stages was the main issue of this study. Ethylene- dependent parameters like colour, chlorophyll (SFR_R index) and lycopene are influenced by NOP-1 in the ripening stages of MG and BR (Lelievre et al., 1997). Softening, which underlies a two-pathway control of developmental and ethylene- dependent regulation, was not impaired by NOP-1 at any application time and modus. (Pech et al., 2008; Sitrit and Bennett, 1998). Based on the assumption that the amount of blocked ethylene is critical as to how much this blockage affects the inhibition of the respective parameter, it seems that the firmness could only

be influenced by an almost complete inhibition of ethylene (Pech et al., 2008; Sitrit and Bennett, 1998). In contrast to NOP-1, 1-MCP strongly supresses autocatalysis of ethylene and might therefore lead to a more pronounced ripening delay (Alexander, 2002; Bisson et al., 2016; Lelievre et al., 1997). Due to the reversible binding of NOP-1 to ETR1, a repeated application was expected to enhance ripening delay, an effect that could not be confirmed in our study (Fig. 1). Application of NOP-1 at maturity stage BR led to a similar ripening delay like application at MG (Figs. 1 and 2 A, C), assuming that posttranslational enzyme activity of the ripening enzymes can be influenced by inhibition of ethylene signalling pathway during the already started maturity process. Pharmacokinetic properties of the peptide in the fruit with long-lasting inhibition of the ETR1-EIN2 interaction seem to give a possible explanation of the missing additional effect of the second NOP- 1 application seven days after the first treatment. A long-term stability of NOP-1 under physiological conditions has been shown. (Bisson et al., 2016).

NOP-1 did not lead to significant effects on maturation when applied at the advanced maturity stage PI (Fig. 2 B). A stage-dependent effect has also been observed for 1-MCP and nitric oxide application with weaker inhibition of maturation in advanced stages of ripeness, such as pink and light red (Eum et al., 2009; Hurr et al., 2005; Mir et al., 2004). The results presented here suggest that 1-MCP might cause impairments in colour development while NOP-1 has no detectable effect on ripening delay at stage PI (Hurr et al., 2005; Mir et al., 2004; Poyesh et al., 2018). The advantage of NOP 1 over 1-MCP is the ease of application in aqueous solution as spray or bath despite the weaker and shorter effect. Therefore, it is suitable for on demand use by retailers, regardless of the storage environment of the fruit. Use in the preharvest setting is also conceivable to synchronize the maturity without any longer terms restraint.

In conclusion, the inhibition of the ethylene signalling pathway by means of protein-protein interaction through the octapeptide NOP-1 results in a ripening delay of tomatoes when applied at early maturity stages. These results suggest that NOP-1 offers new opportunities to control climacteric fruit ripening with ease of use as an aqueous solution. Repeated application at the early maturity stage and application at advanced ripening stages had no additional, respectively any effect at all. The NOP-1 influenced parameters suggest that NOP-1 inhibit the ethylene-dependent ripening process. The Further investigations on the efficacy spectrum and properties of NOP-1 are mandatory to explore the scientific and commercial potential of NOP-1.

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E Summary and conclusion

The objective of this study was to evaluate the mode-of-action, and proof the concept, of the ripening inhibitor peptide NOP-1 and its impact on external and internal quality attributes of tomatoes and apples. To evaluate the ripening delaying effect of NOP-1 basic experiments were done by treating harvested tomato in the maturity stage 'mature green'. Solution droplets were applied at different concentrations to create concentration-response-curves and time-response-curves. Ripening parameters and quality attributes were measured with traditional methods and non-destructive parameters recorded by optical sensors.

In a second series of experiments, NOP-1 was applied on postharvest to Golden Delicious apples in a concentration of 1000 µM, which was the most effective concentration on tomatoes. Here 1-MCP treated fruits were used as reference with regard of ripening delay, quality changes, ethylene release and respiration during cold process. The aim was to investigate the transferability of the NOP-1 induced maturity delay in tomatoes of apples, which produce many times more ethylene as tomatoes, applied in two different ways.

In a third approach, the ripening inhibition by NOP-1 was studied when it was applied to different maturity stages of tomatoes, and repeated applications, to investigate the maturity stage-dependent efficacy of the peptide, and the possibility of enhancing the efficacy.

In the following, the most relevant results of the conducted investigations are presented.

1. A concentration of 1000 µM of the NOP-1 solution showed the most potent ripening inhibiting effect in tomatoes compared with concentrations of 400 µM and 2000 µM. The duration of the maturity delay was about 7 days as indicated by hue index, the chlorophyll degradation and lycopene and ß-carotene accumulation. Fruit firmness was not affected by NOP-1. There was no quality impairment of treated fruits regardless of the peptide concentration as compared to untreated fruits.

2. NOP-1 led to a small extension of the shelf-life of apples when applied with brush. Colour development and chlorophyll degradation were delayed by 2 to 3 days. The effect of peptide application as microdroplets was weaker. Flesh

firmness, starch degradation, titratable acidity and total soluble solids were not affected by NOP-1. 1-MCP significantly delayed all ripening parameters and strongly suppressed ethylene production and respiration activity. In contrast, NOP-1 did not have any effect in inhibiting ethylene release or respiration rate during shelf-life. There were no differences in the evaluated quality parameters.

3. Application of NOP-1 on tomato at the maturity stage breaker induced a similar ripening delay as the application in the maturity stage green, by slowing down the colour change (hue index) for about seven days. The SFR_R chlorophyll fluorescence index even showed a slowdown in the decline of 21 days compared to the untreated controls. In parallel, there was a delay in the accumulation of lycopene. Firmness was not affected by the peptide application. Further, chlorophyll content and ß-carotene concentration did not differ from control fruits after reaching fully ripe stage. Repeated application had no additional delaying effect and did not reach the ripening delay induced by 1-MCP. Peptide application at maturity stage pink had no maturity inhibitory effect at all.

In general, the recombinant octapeptide NOP-1 delayed ripening of tomatoes and apples when applied as aqueous solution. The peptide-induced inhibition of ETR – EIN2 complex formation impairs ethylene signalling and offers the opportunity for a biological approach of ripening control of climacteric fruits, as shown here on tomatoes and apples.

From the studies, it is clear that different ripening and quality parameters have been influenced in different ways and/or magnitudes. While changes in colour and dynamics of chlorophyll and other pigments were more affected, alterations in the content of sugar and acids, or alterations in fruit firmness, were less or not affected. These reactions indicate that different steps in the ripening might be affected. The common aspect is that changes in many quality attributes such as changes in pigment contents, respiration rate, softening of fruit flesh are triggered by ethylene. However, multiple enzymatic and metabolic steps are involved and responsible for each quality attribute. Furthermore, each process might undergo a different temporal dynamic, and might also be triggered by different ethylene levels. Finally, particularities of plant species, varieties, maturation stage of the fruits, history of the fruit during its growth and developmental phases, physicochemical properties of the fruits, application method, application timing and concentration of the ripening inhibitor, may play a significant role concerning the efficacy of the proposed ripening inhibition method.

The different compositions of the cuticle of apple and tomato and the related compositional diversity in cuticular waxes may account for the reduced effect of NOP-1 on ripening in apples compared with tomatoes. The lower affinity of the NOP-1 peptide to the apple ethylene receptor (MdETR1) in comparison to the tomato ETR (LeETR1) may also contribute to the difference between apples and tomatoes. In addition, it is important to consider the variation in ethylene production and ethylene sensitivity between apple and tomato. In this case, the higher ethylene production rate and higher ethylene sensitivity in apple could be another reason to explain the weaker delaying effect of NOP-1. NOP-1 is a competitive inhibitor of EIN2 – ETR interaction which acts downstream of receptor activation and does not impair ethylene autocatalysis. It is assumed that the reversible inhibition of EIN2 – ETR interaction does not affect all ethylene response functions like ethylene-dependent changes in fruit firmness.

With our studies we demonstrate on tomatoes and apples the efficacy of NOP-1 in delaying the ripening of fruits, as determined for specific quality parameters. Further studies have to follow by considering all the topics mentioned above. In addition, dose-response studies under other storage conditions should be performed. In particular, it should be determined how is the cuticular penetration of the NOP-1 peptides into the fruits, a necessary step to ensure its effect in the inner part of the fruits. Specially, penetration amount should be quantified, and checked, if it can be improved through proper technical formulation of the solution. Such studies are mandatory to finally delineate the potential for commercial adoption of NOP-1.

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