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A novel carotenoid, 4-keto-α-carotene, as an unexpected by-product during genetic engineering of carotenogenesis in rice callus

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ABSTRACT

Rice endosperm is devoid of carotenoids because the initial biosynthetic steps are absent. The early carotenogenesis reactions were constituted through co-transformation of endosperm-derived rice callus with phytoene synthase and phytoene desaturase transgenes. Subsequent steps in the pathway such as cyclization and hydroxylation reactions were catalyzed by endogenous rice enzymes in the endosperm. The carotenoid pathway was extended further by including a bacterial ketolase gene able to form astaxanthin, a high value carotenoid which is not a typical plant carotenoid. In addition to astaxanthin and precursors, a carotenoid accumulated in the transgenic callus which did not fit into the pathway to astaxanthin. This was subsequently identified as 4-keto-α-carotene by HPLC co-chromatography, chemical modification, mass spectrometry and the reconstruction of its biosynthesis pathway in *Escherichia coli*. We postulate that this keto carotenoid is formed from α-carotene which accumulates by combined reactions of the heterologous gene products and endogenous rice endosperm cyclization reactions.

Introduction

Genetic engineering is a powerful tool to modulate existing biosynthesis pathway or establish novel routes in microbes and plants. Carotenogenesis is a key target for genetic engineering of staple crops due to the many nutritional and other health benefits of a number of such molecules for humans and animals (Fraser et al., 2009; Misawa, 2011). Successful examples are increase of the carotenoid yield, e.g., lycopene in tomato (Fraser et al., 2002), the accumulation of intermediates to higher levels, e.g., zeaxanthin in potato (Römer et al., 2002) or extension of an existing pathway to a novel end product, e.g., astaxanthin in maize (Zhu et al., 2008). In addition, maize was used to explore interactions between an induced transgenic carotenoid pathway and the endogenous pathway (Naqvi et al., 2011). On a number of occasions there have been examples of novel unexpected phenotypes due to unknown regulatory mechanisms and unpredicted enzyme interactions (see Sandmann et al. (2006) for review). Rice provides an interesting example of carotenogenesis by genetic engineering. In contrast to the endosperm of yellow maize which is pigmented due to the accumulation of lutein, zeaxanthin, and 5,6-epoxy derivatives Quackenbush et al. (1963)), rice endosperm is colourless. Nevertheless, rice endosperm possesses a hidden potential for carotenoid biosynthesis even though the initial steps in the carotenoid pathway are absent. The endogenous levels of phytoene synthase and phytoene desaturase in wild type endosperm are below the threshold level for carotenoid biosynthesis (Schaub et al., 2005). It has been demonstrated that the limitation of carotenogenesis can be overcome in rice endosperm by expressing genes encoding a phytoene synthase and a bacterial phytoene desaturase able to replace all plant desaturation and isomerisation reactions (Ye et al., 2000). The expected product of these reactions is lycopene. Interestingly biosynthesis proceeded beyond lycopene by cyclization to α- and β-carotene and the hydroxylation of both carotenes to lutein and zeaxanthin, respectively (Ye et al., 2000). Thus intrinsic rice cyclases and hydroxylases are expressed in the endosperm. A survey of phytoene synthase genes from different plant species indicated that the maize enzyme is the most effective in rice (Paine et al., 2005). Its use led to the generation of a rice line rich in α- and β-carotene which both exhibit provitamin A activity, in addition to lutein and zeaxanthin (structures shown in Fig. 1). In our current carotenogenesis engineering experiments we attempted to extend the pathway beyond carotenes to astaxanthin. Astaxanthin is a high priced carotenoid which is beneficial for human health

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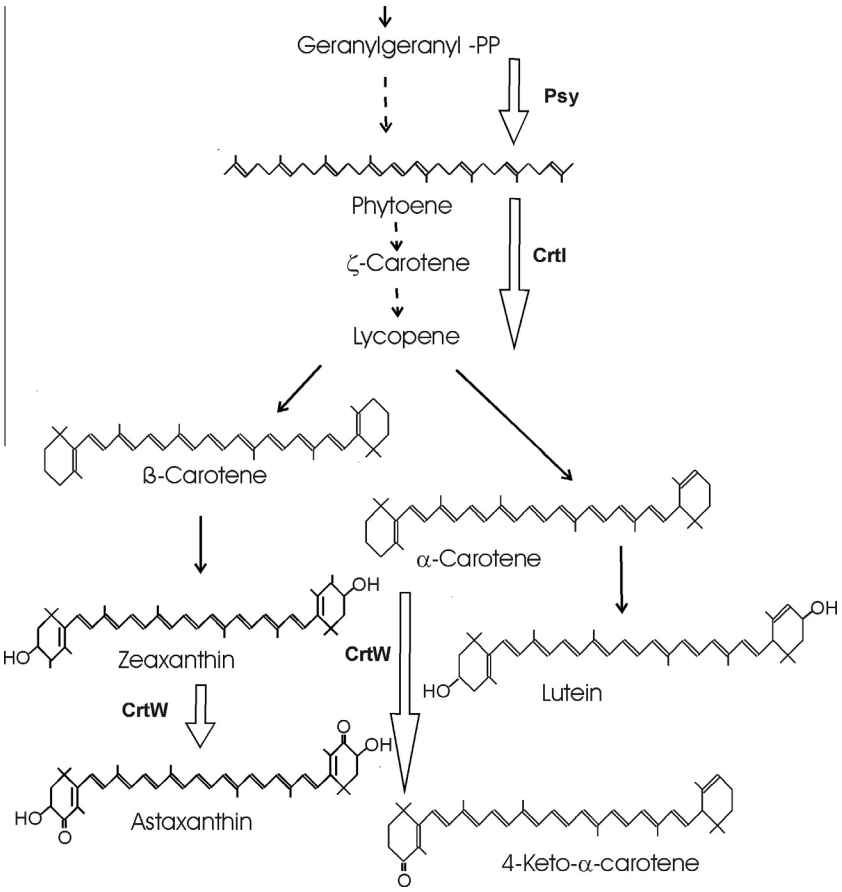

Fig. 1. The biosynthesis pathway of astaxanthin formation in transgenic endosperm-derived rice callus. Dotted arrows indicate pathway limitations in rice, open arrows indicate the reactions catalaysed by the transgenes *psy* (phytoene synthase), *crtI* (phytoene desaturase) and *crtW* (carotene ketolase), solid arrows indicate maize-specific carotenogenic reactions.

(Guerin et al., 2003; Hussein et al., 2006) and is used as an essential feed additive in salmon farming (Bjerkeng, 2008).

Considerable effort has been invested to achieve sustainable and economic production of astaxanthin in microorganisms and plants (Zhu et al., 2009). Our strategy has been to engineer suitable plant material such as rice grains to synthesize astaxanthin for directly feeding or consumption (Sandmann, 2001). In previous work, we reported that transformation of maize with a ketolase and an additional hydroxylase gene resulted in the accumulation of astaxanthin (Zhu et al., 2008). However, the yields were not satisfactory. Therefore, we have chosen rice as an alternative crop for the engineering of carotenogenesis using combinatorial genetic transformation (Farre et al., 2012). The endosperm-based rice callus system allowed us to investigate the impact of genetic pathway modulation on carotenoid composition before the time consuming and labour intensive regeneration of intact plants. Transformation was carried out simultaneously with three genes, a maize phytoene synthase, a bacterial phytoene desaturase and a bacterial β-carotene ketolase gene which in combination with the endogenous endosperm expressed β-carotene hydroxylase should be able to synthesize astaxanthin.

Engineering of carotenoid biosynthesis in rice endosperm was successful in terms of engineering a ketolation pathway leading to astaxanthin as the end product. However in addition to the expected hydroxy and keto intermediates derived from β-carotene, we detected a novel carotenoid which did not fit directly into the pathway to astaxanthin. Its identification is the scope of this work.

Results and discussion

The initial synthesis of phytoene and its 4-step desaturation and isomerization to lycopene was engineered into rice callus in combination with a bacterial carotenoid ketolase (Fig. 1). Expression of carotenoid ketolase and all other transgenes has been shown previously (Bai et al.2013). Fig. 2A shows the HPLC carotenoid profile of a typical transgenic line. Two carotenes were synthesized by the interaction of phytoene synthase and desaturase and the endogenous lycopene cyclases, α-carotene (peak 5) and β-carotene (peak 6). The prominent carotenoid peak 1 at 6.7 min with the typical bell shaped optical absorbance spectrum with its maximum at 475 nm resembles astaxanthin. This is the end product of the ketolation pathway starting from β-carotene or zeaxanthin (Fig. 1) which was initiated by the transgenic carotenoid ketolase. This enzyme works by interaction with the endogenous β-carotene hydroxylase which is active and specific enough for astaxanthin synthesis. Two other peaks resemble intermediates of this pathway, 4-keto-zeaxanthin peak 2 and echinenone peak 4, both with an asymmetrical bell shaped optical absorbance spectrum with a maximum at 465 nm (Fig. 3B). The latter originated directly from ketolation of β-carotene, the other by ketolation of zeaxanthin.

One compound which does not fit into this pathway is the carotenoid represented by peak 3 (Fig. 2A). Its spectrum is shown in Fig. 3A. Although it has a very similar shape to the spectrum of echinenone (Fig. 3B) which may indicate a mono-keto carotenoid, its maximum of 452 nm is 14 nm lower than that of echinenone. The only known carotenoid with the same absorbance and

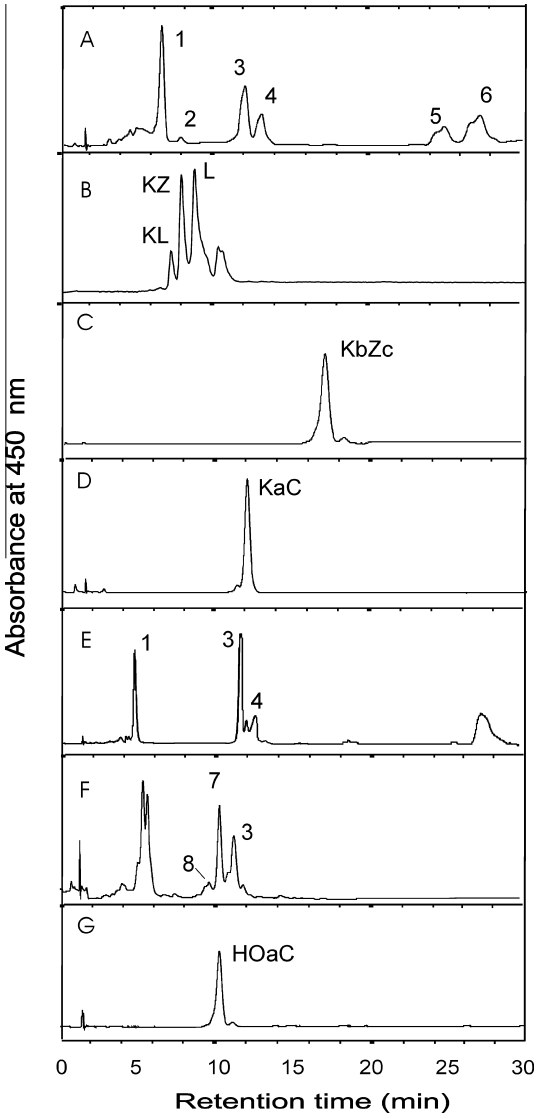

Fig. 2. HPLC separation of carotenoids from transgenic rice callus expressing a ketolase gene (A). (B) isolate with 4-keto-lutein, (C) 4-keto-β-zeacarotene (KbZc) standard, (D) 4-keto-α-carotene standard (KaC), (E) co-chromatography of rice extract with 4-keto-α-carotene standard, (F) carotenoids from transgenic rice callus after reduction with $NaBH_4$, (G) 4-hydroxy-α-carotene (HOaC) standard. Peak 1, astxanthin; 2, 4-keto-zeaxanthin; 3, novel unknown carotenoid; 4, echinenone; 5, α-carotene; 6, β-carotene; 7, 4-hydroxy-α-carotene; 8, 4-hydroxy echinenone. L, lutein; KL, 4-keto-lutein; KZ, 4-keto-zeaxanthin.

shape of the spectrum is 4-ketolutein (Fig. 3C). This was extracted from transgenic *Nicotiana tabaccum* nectary tissue transformed with a cyanobacterial β-carotene ketolase gene (Gerjets et al., 2007). However, it is more polar with a retention time of 7.5 min compared to 12.2 min for peak 3 (Fig. 2B). From the spectrum and the basic carotenoid pathway to α- and β-carotene related compounds, 4-keto-β-zeacarotene (7,8-dihydro-β,ψ-carotene-4-one) and 4-keto-α-carotene (β,ε-carotene-4-one) are the most likely candidates for the novel carotenoid molecule. Therefore, we generated these mono keto carotenoids which should exhibit not only a similar absorbance but also a similar polarity as reference compounds by combinatorial biosynthesis in *Escherichia coli* (Sandmann, 2002). In the case of 4-keto-β-zeacarotene the absorbance maximum was the same as that of the novel compound but the shape of the absorbance peak did not match (Fig. 3D). In addition, this carotenoid did not co-chromatograph with compound 3 due to its longer retention time (Fig. 2C). Only 4-keto-α-carotene matched exactly the spectrum of compound 3 (Fig. 3E), it exhibited the same retention time in HPLC (Fig. 2D) and co-chromatographied with peak 3 (Fig. 2E). For further confirmation of the structure of compound 3, the carotenoid extract from rice was reduced by sodium borohydride. This chemical modification of the keto group was also carried out with the 4-keto-α-carotene reference compound resulting in 4-HO-α-carotene. In each case, a more polar compound was formed (Fig. 2D and F). The spectrum of this hydroxy derivative shows the typical three maxima of non-ketolated carotenoids exhibiting a hypsochromic shift to 442 nm of the main central maximum (Fig. 3F) for reduction of a keto group conjugated with the polyene chain. The resulting spectrum resembles that of α-carotene (Britton et al., 2004).

For further structure elucidation of peak 3, a mass spectrum was determined by UHPLC-MS with atmospheric pressure chemical ionisation identifying the $[M+H]^+$ ions (Fig. 4A). The dominating mass peak in the spectrum is the ion at m/z 552. This may resemble the protonated molecular ion of compound 3 with a molecular mass of 551 g/mol. Several carotenoid masses fit this value including the one for echinenone with 550.86 g/mol (Rivera et al., 2011). Starting from $[M+H]^+$ ion of 552, transitions related to functional groups in the carotenoid structures were built. The ones shown in Fig. 4B were those with characteristic signals for identification. The ones to identify an echinenone-related structure are the transitions 551.6 > 203.1 resulting from the cleavage at C-10,11 of a 4-keto fragment as indicated in Fig. 4C (Van Breemen et al., 2012), transition 551.6 > 93 by in-chain elimination of toluene and transition 551.6 > 69 previously reported for echinenone (Enzell et al., 1969). One of the most intense transitions is 551.6 > 123.1. This transition is a specific indicator of the existence of an ε-ring in the structure of compound 3 (Enzell and Back, 1995). Taken together results from mass spectrometry suggest that compound 3 is a carotenoid combining the ketolated half of echinenone and the other half with the ε-ring of α-carotene (boxed in Fig. 4C). This is consistent with 4-keto-α-carotene which has the same molecular mass as echinenone.

HPLC co-chromatography, chemical modification and mass spectrometry all identified compound 3 as 4-keto-α-carotene. This is a unique carotenoid not reported thus far (Britton et al., 2004). A similar but different carotenoid is 2-keto-α-carotene from the stick insect *Ectatosoma tiaratum* (Kayser, 1981). Due to the position of the keto group at C-2, it does not contribute to the conjugated polyene chain which influences the optical absorbance spectrum with three pronounced peaks at 423, 446 and 475 nm, distinct from the spectrum in Fig. 3E. Formation of 4-keto-α-carotene is due to the relatively high α-carotene content in the transgenic rice callus. Obviously, the carotene ketolase from *Brevundimonas* sp. possesses broad substrate specificity. This enzyme typically ketolates both β-ionone rings in β-carotene (Nishida et al., 2005). In addition, it is also able to ketolate the β-ionone end of the α-carotene molecule. This broad substrate specificity in combination with the α-carotene concentrations as substrate for the ketolase is the primary reason for the formation of 4-keto-α-carotene in the genetically engineered rice callus.

Conclusion

Although genetic engineering of the carotenoid pathway to astaxanthin was successful, a specific side reaction occurred in rice callus. The expressed bacterial ketolase not only converted β-carotene and zeaxanthin to astaxanthin but also accepted the β-ionone ring of α-carotene as substrate for ketolation. The resulting product was identified as 4-keto-α-carotene. Its formation branches off the desired β-carotene to astaxanthin route. This loss for the

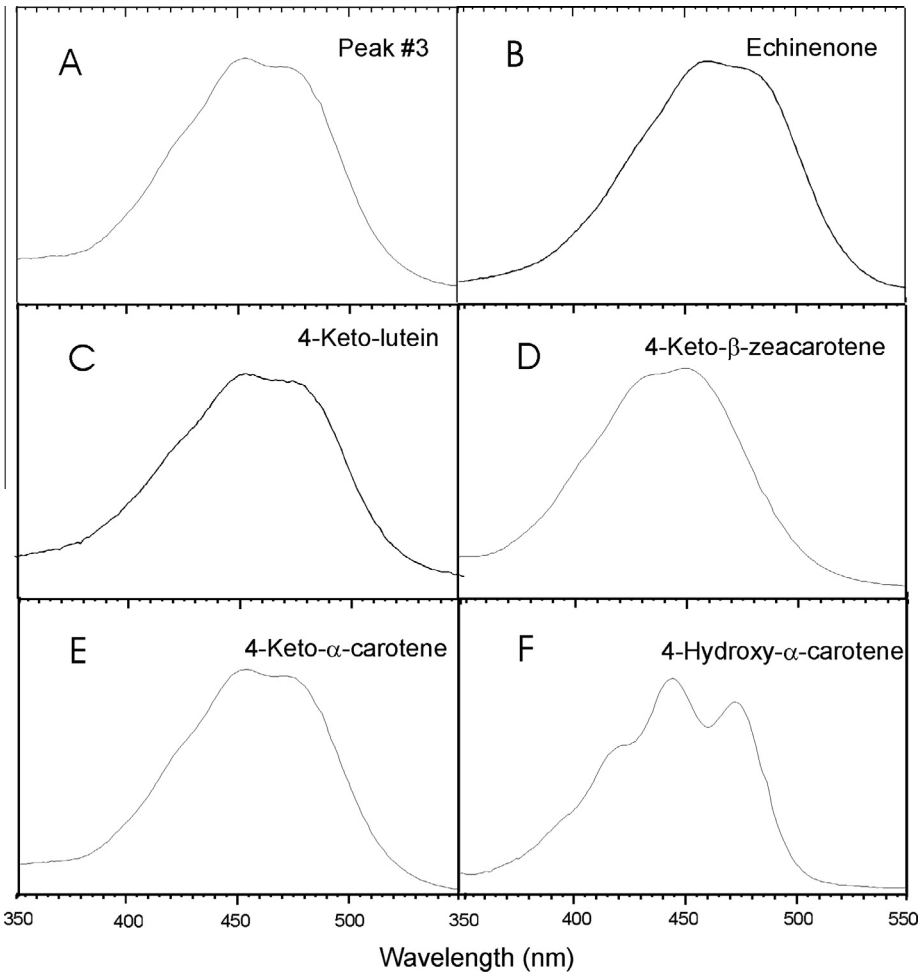

Fig. 3. Optical spectra of the novel unknown carotenoid (A), echinenone (=4-keto-β-carotene) (B), 4-keto-lutein (C), 4-keto-β-zeacarotene (D), 4-keto-α-carotene (E) and 4-hydroxy-α-carotene (F).

astaxanthin pathway may be avoided by further genetic engineering of carotenogenesis by antisense or RNAi down-regulation of the expression of rice specific lycopene ε-cyclase depleting the formation of α-carotene.

Experimental

Transgenic rice

The transgenic endosperm-derived rice callus was generated by combinatorial nuclear transformation which has been reported earlier (Zhu et al., 2008). The three plasmids contained phytoene synthase *Zmpsy1*, phytoene desaturase *Pacrtl* and β-carotene ketolase CrtW from *Brevundimonas* sp. Strain SD212 (Nishida et al., 2005). The latter gene was chemically synthesized according to the codon usage of *Brassica napus* and fused to the full-length rice alcohol dehydrogenase 5́-untranslated region (Sugio et al., 2008) and to the transit peptide sequence from pea ribulose-1, 5-bisphospate carboxylase small subunit (Schreier et al., 1985). This DNA fragment was inserted into plasmid GZ63 containing the maize γ-zein gene promoter and the *nos* terminator. The maize *psy1* cDNA (Buckner et al., 1996) was transferred into plasmid p326 containing the wheat LWM glutenin promoter (Stoger et al. 1999) and *nos* terminator. The *crtI* gene from *Pantoea ananatis* (formerly known as *Erwinia uredovora*) *was* fused in frame with the transit peptide signal from the *Phaseolus vulgaris* small subunit of ribulose bisphosphate carboxylase in plasmid pYPIET4 (Misawa et al., 1994), amplified by PCR and then transferred to pHorp-P containing the barley D-hordein promoter (Sørensen et al., 1996) and the rice ADPGPP terminator. All transformation constructs are based on pUC8 plasmids. Their maps are shown in Fig. 5. Transgenic coloured calli were selected and cultured on MS selection medium (Farre et al., 2012).

Combinatorial carotenoid synthesis in E. coli

For the synthesis of reference compounds the biosynthesis pathways for 4-keto-α-carotene and 4-keto-β-zeacarotene the following combinations of compatible plasmids were used to transform *E. coli* strain DH5α. Plasmid pACCAR16ΔcrtX (Misawa et al., 1995) in combination with pBBRK-ara-epsilon with the lycopene ε-cyclase from *Arabidopsis thaliana* (Cunningham et al., 1996) cloned into pBBR1-MCS1 with kanamycin as selection marker (Kovach et al., 1994) mediates the formation of α-carotene. This carotene is then ketolated at the β-ionone ring to 4-keto-α-carotene by expression of pPEU30crtO (Breitenbach et al., 2013). 4-Keto-β-zeacarotene is synthesized from neurosporene generated by pACCRT-EBI$_{RC}$ (Linden et al., 1993) which is cyclised by expression of plasmid pRK-crtY (Hausmann and Sandmann, 2000) and finally ketolated by expression of pCRBKT (Zhong et al., 2011). The *E. coli* transformant generating 4-keto-α-carotene was grown at 37 °C in the presence of ampicillin (100 μg/ml), chloramphenicol

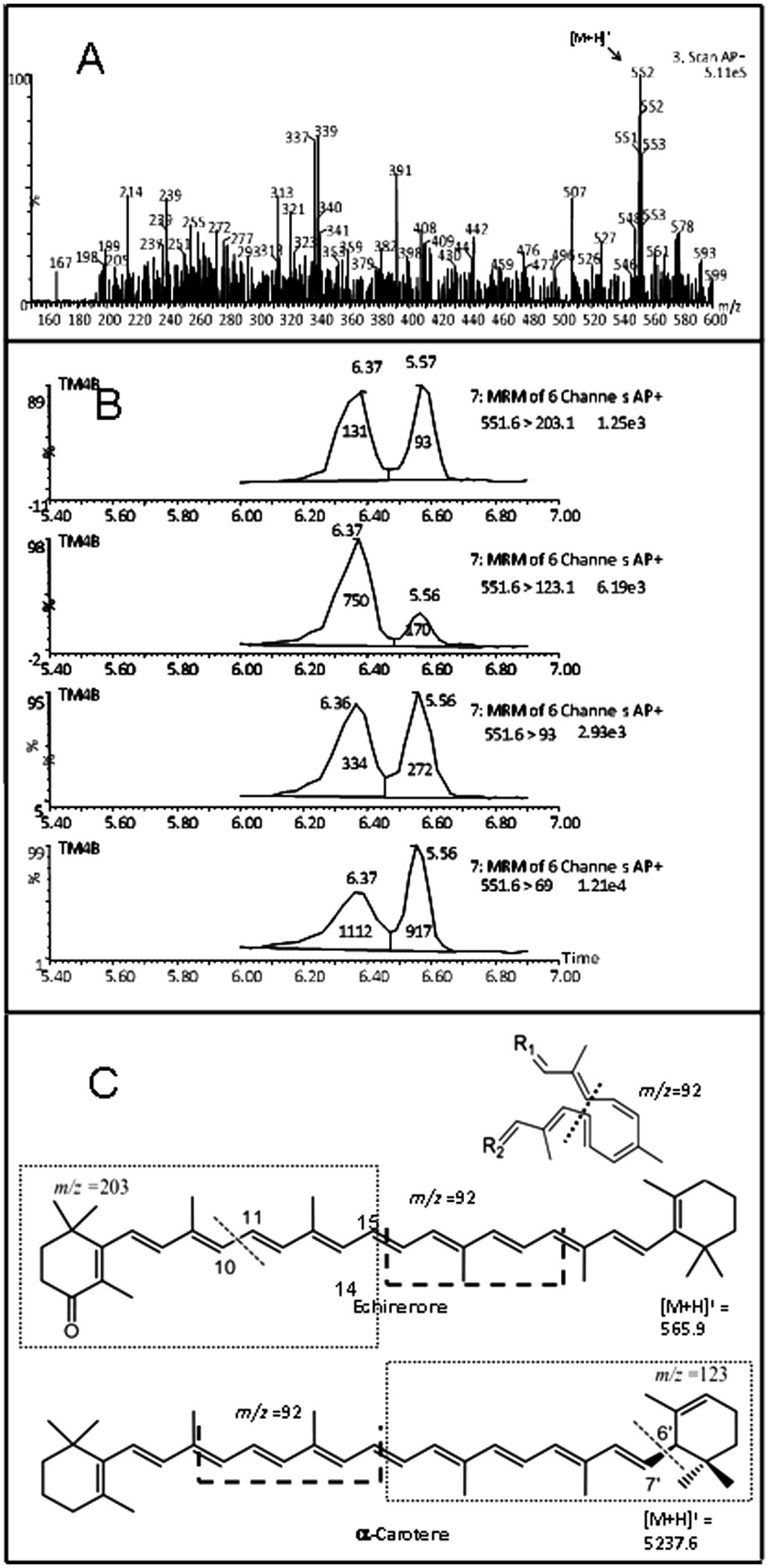

Fig. 4. Mass spectroscopy of novel unknown compound. (A) Atmospheric pressure chemical ionisation mass spectrum, (B) transitions from the $[M+H]^+$ ion of 552, (C) fragmentation pattern of echinenone and α-carotene. Boxes indicate the two halves of the identified molecule, 4-keto-α-carotene.

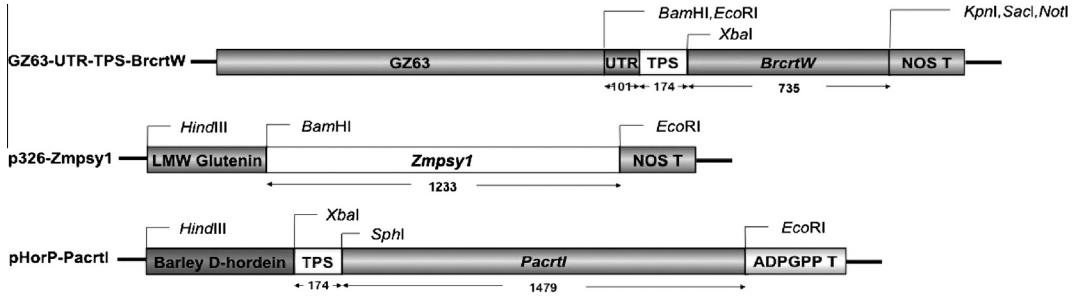

Fig. 5. pUC8-derived plasmid constructs used for rice transformation to express carotene ketolase (BrcrtW), phytoene synthase (Zmpsy1) and phytoene desaturase (PacrtI)). GZ63, maize γ-zein gene promoter; UTR, 5-untranslated region from rice alcohol dehydrogenase; TPS, transit peptide from pea ribulose-1, 5-bisphospate carboxylase small subunit; NOS T, *nos* terminator; LMW Glutenin, LWM glutenin promoter; Barley D-hordein, barley D-hordein promoter; ADPGPP T, rice ADPGPP terminator.

(34 μg/ml), kanamycin (25 μg/ml) and IPTG (1 mM) in LB medium (Sambrook et al., 1989). The 4-keto-β-zeacarotene producing transformant was cultured at 28 °C in the same medium without IPTG replacing kanamycin with tetracyclin (25 μg/ml).

Carotenoid extraction and analysis

Carotenoids were extracted from freeze-dried rice callus, *E. coli* cells and nectary tissue from *N. tabaccum* (Gerjets et al., 2007) for 20 min with methanol at 60 °C and partitioning into 10% ether in petrol. In the case of tobacco nectary tissue, the 10% ether in petrol phase was diluted 3:1 with hexane, passed through a silica column and the adsorbed polar carotenoid fraction eluted with acetone (Steiger et al., 1999). Analytical HPLC was isocratic on a 15 cm Nucleosil C18, 3 μ column with acetonitrile/ methanol/2-propanol (85:10:5, v/v) at 10 °C and a flow 0.8 ml/min. Carotenoids were identified by their optical absorbance spectra and co-chromatography with standard carotenoids. In the case of the unknown carotenoid, its biosynthetic pathway was reconstructed in *E. coli*. This carotenoid was reduced to 4-hydroxy-α-carotene with sodium borohydride (Eugster, 1995). For mass analysis, UHPLC was carried out using an ACQUITY Ultra Performance LC TM system linked to an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). MassLynx™ software version 4.1 (Waters, Milford, MA, USA) was used to control the instruments, and also for data acquisition and processing. Separation was on a reverse phase ACQUITY UPLC® C18 BEH 130 Å, 1.7 mm, 2.1 × 100 mm column (Waters, Milford, MA) and a gradient system with the mobile phase consisting of solvent A: acetonitrile:methanol (70:30, v/v) and solvent B: water 100% at a flow rate of 0.35 ml/min. (Rivera et al., 2013).

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