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Biosynthesis of scopoletin and scopolin in cassava roots during post-harvest physiological deterioration: The *E*-*Z*-isomerisation stage

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ABSTRACT

Two to three days after harvesting, cassava (*Manihot esculenta* Crantz) roots suffer from post-harvest physiological deterioration (PPD) when secondary metabolites are accumulated. Amongst these are hydroxycoumarins (e.g. scopoletin and its glucoside scopolin) which play roles in plant defence and have pharmacological activities. Some steps in the biosynthesis of these molecules are still unknown in cassava and in other plants. We exploit the accumulation of these coumarins during PPD to investigate the *E*-*Z*-isomerisation step in their biosynthesis. Feeding cubed cassava roots with *E*-cinnamic-3,2′,3′,4′,5′,6′-d_5 acid gave scopoletin-d_2. However, feeding with *E*-cinnamic-3,2′,3′,4′,5′,6′-d_6 and *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acids, both gave scopoletin-d_3, the latter not affording the expected scopoletin-d_4. We therefore synthesised and fed with *E*-cinnamic-2-d_1 when unlabelled scopoletin was biosynthesised. Solely the hydrogen (or deuterium) at C2 of cinnamic acid is exchanged in the biosynthesis of hydroxycoumarins. If the mechanism of *E*-*Z*-cinnamic acid isomerisation were photochemical, we would not expect to see the loss of deuterium which we observed. Therefore, a possible mechanism is an enzyme catalysed 1,4-Michael addition, followed by σ-bond rotation and hydrogen (or deuterium) elimination to yield the *Z*-isomer. Feeding the roots under light and dark conditions with *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid gave scopoletin-d_3 with no significant difference in the yields. We conclude that the *E*-*Z*-isomerisation stage in the biosynthesis of scopoletin and scopolin, in cassava roots during PPD, is not photochemical, but could be catalysed by an isomerase which is independent of light.

1. Introduction

Cassava (*Manihot esculenta* Crantz Family Euphorbiaceae) is an important economical and nutritional crop, the fourth most important food source in tropical countries due to its high root starch content. Although cassava is relatively easy to grow, even in poor soils and under drought conditions, its roots have a short shelf-life of only one to three days due to post-harvest physiological deterioration (PPD) which can cause significant wastage and economic losses. Within 2–3 days of harvesting, the roots show blue to black vascular streaking and are unpalatable and therefore unmarketable, significantly affecting the crop's economic value. PPD has been explained as a physiological process not due to microorganisms, (Averre, 1967; Noon and Booth, 1977) and on a molecular basis as an oxidative burst which initiates within 15 min of the root being injured, (Reilly et al., 2003, 2004) followed by altered gene expression. This genetic change is predicted to play roles in cellular processes including: reactive oxygen species turnover, cell wall repair, programmed cell death, ion, water or metabolite transport, signal transduction or perception, stress response, metabolism and biosynthesis, activation of protein synthesis (Reilly et al., 2007) and the accumulation of secondary metabolites (Tanaka et al., 1983; Buschmann et al., 2000). Amongst these secondary metabolites are hydroxycoumarins (e.g. scopoletin and its glucoside scopolin) which show antioxidant properties and which may by oxidation and polymerisation give rise to the blue/black discolouration. These hydroxycoumarins are important in plant defence as phytoalexins due to the induction of their biosynthesis following various stress events (wounding, bacterial and fungal infections) (Giesemann et al., 1986; Gutierrez et al., 1995). Additionally, they display a wide range of pharmacological activities, including anti-coagulant (Mueller, 2004), anti-inflammatory (Silvan et al., 1996), antimicrobial (Valle et al., 1997; Cespedes et al., 2006) and anticancer (Kawaii et al., 2001; Kawase et al., 2003; Lacy and O'Kennedy, 2004) activities. However, their biosynthesis in cassava is not known and neither is it clearly understood in other plants (Petersen et al., 1999).

Indeed, the biosynthesis of coumarins in plants is not well understood, although these metabolic pathways are often found in the plant kingdom. From biosynthetic studies in *Arabidopsis thaliana* ecotype Columbia, Kai et al. (2006) recently reported in this

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Journal on the occurrence of high levels of the coumarins scopoletin and its β-D-glucopyranoside, scopolin, found in the wild-type roots 180-fold higher than in aerial parts. Their studies with mutants led to the identification of 3′-hydroxylation of *p*-coumarate catalysed by CYP98A3, one of the few enzymes to be identified unambiguously along this complex phenylpropanoid pathway. More recently in *A. thaliana*, the same research group showed that a Fe(II)- and 2-oxoglutarate-dependent dioxygenase, rather than a cytochrome P450 enzyme, catalyses the *o*-hydroxylation of feruloyl-CoA in scopoletin biosynthesis (Kai et al., 2008).

As part of on-going studies into these hydroxycoumarins (Buschmann et al., 2000; Reilly et al., 2003, 2004), we investigated the incorporation of cinnamic acid-d_7 into cassava roots under PPD, in order to prove that it was a potential precursor of scopoletin and scopolin on the phenylpropanoid pathway (Fig. 1), from phenylalanine following the action of phenylalanine ammonia lyase (PAL).

The unexpected experimental result that scopoletin-d_3 was produced, and not scopoletin-d_4 (Fig. 2), has led us to study in detail the *E*-*Z*-isomerisation step in the biosynthesis of scopoletin and scopolin. This isomerisation is not resolved in plants, and has not been previously reported in cassava under PPD. Here, we exploit the increase in hydroxycoumarin accumulation in cassava roots post-harvest to investigate this isomerisation step in the biosynthesis of scopoletin and scopolin.

1.1. The E-Z-cinnamic acid isomerisation stage in different plants

In order to interpret this unexpected loss of deuterium at position 2 when *E*-cinnamic-d_7 acid was fed to cassava root under PPD, we undertook a detailed literature search on previous studies of the *E*-*Z*-isomerisation step in coumarin biosynthesis. These report that the mechanism of this step varies between genera and even between species. It has been reported (Edwards and Stoker, 1967, 1968) that the isomerisation may be induced by UV light in vivo, as has been demonstrated in vitro (Koenigs et al., 1993; Zheng et al., 1999). Photoisomerisation of the *E*-double bond, in *p*-coumarate, has been studied using yellow protein that ultimately mediates a phototactic response to blue light in certain purple bacteria *Ectothiorhodospira* (Ryan et al., 2002; Dugave and Demange, 2003). Feeding *Melilotus officinalis* shoots with *E*-*o*-coumaric acid-2-^{14}C in both dark and light conditions showed much more radioactivity in the coumarins isolated from shoots exposed to light than in the coumarins isolated from shoots kept in the dark. Edwards and Stoker therefore concluded that an isomerase enzyme is not involved in the isomerisation of *o*-coumaric acid in *M. officinalis* shoots (Edwards and Stoker, 1967). In lavender (*Lavandula officinalis* and *L. spica*), the biosynthesis of herniarin (7-methoxycoumarin) has been shown to be non-enzymatic, and the reaction is light catalysed. Therefore, based on the available evidence in 1967, Edwards and Stoker concluded that "it is probable therefore that the isomerisation step in the biosynthesis of all plant coumarins is entirely photochemical" (Edwards and Stoker, 1968). More recently, in this *Journal*, the isomerisation of cinnamic acid derivatives in barley and wheat was "directly attributed to the effect of light, and not apparently modulated by any enzymic reactions" (Turner et al., 1993), and in *A. thaliana* "sunlight was able to isomerise both cinnamic acid isomers" (Wong et al., 2005).

M. alba (sweet clover) plants fed with *E*-cinnamic acid-3-^{14}C in the dark produced ^{14}C-labelled coumarin. The amount of radioactivity in the coumarin was less than when plants were exposed

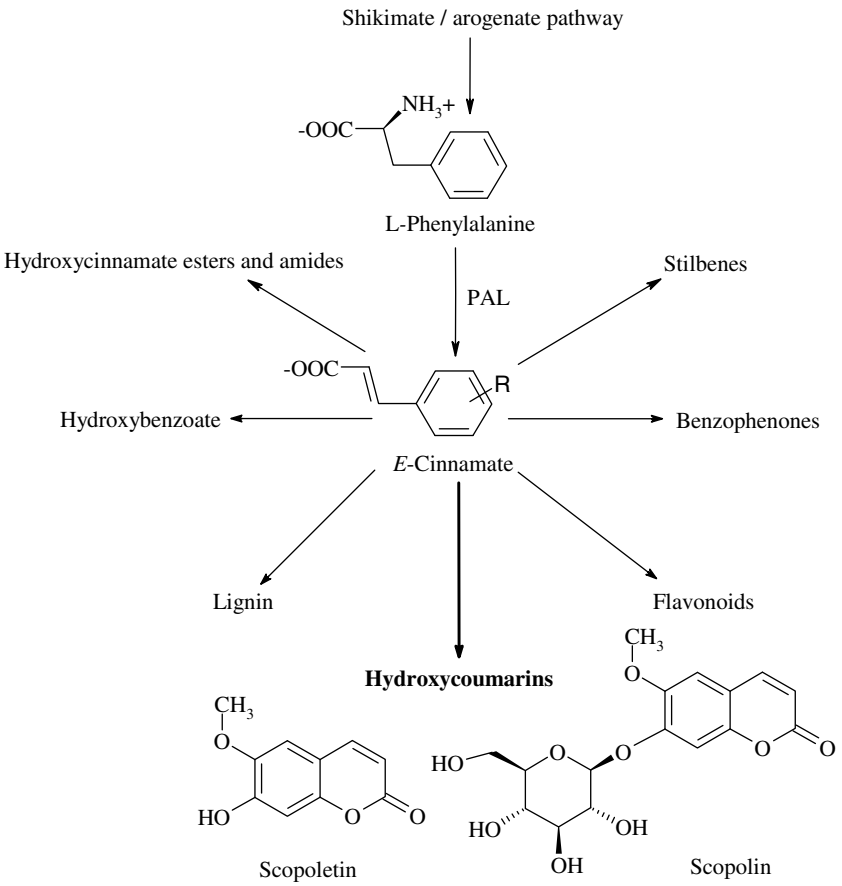

Fig. 1. Biosynthetic pathways of phenylpropanoids.