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Research article

Detection of a plant enzyme exhibiting chlorogenate-dependant caffeoyltransferase activity in methanolic extracts of arbuscular mycorrhizal tomato roots

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ABSTRACT

When *Glomus intraradices*-colonised tomato roots were extracted in methanol at 6 °C, chlorogenic acid (5-caffeoylquinic acid), naturally present in the extract, was slowly converted by transesterification into methyl caffeate. The progress of the reaction could be monitored by HPLC. The reaction only occurred when the ground roots were left in contact with the hydro-alcoholic extract and required the presence of 15–35% water in the mixture. When the roots were extracted in ethanol, chlorogenic acid was transformed to ethyl caffeate in the same conditions. The reaction was also detected in *Glomus mosseae*-colonised tomato root extracts. It was also detectable in non-mycorrhizal root extracts but was 10–25 times slower. By contrast it was undetectable in extracts of the aerial parts of tomato plants, which also contain high amounts of chlorogenic acid, whether or not these plants were inoculated by the arbuscular mycorrhizal fungus. We found that this transesterification reaction is catalysed by a tomato enzyme, which remains active in hydro-alcoholic mixtures and exhibits chlorogenate-dependant caffeoyltransferase activity in the presence of methanol or ethanol. This transferase activity is inhibited by phenylmethanesulfonyl fluoride. The 4- and 3-caffeoylquinic acid isomers were also used as substrates but were less active than chlorogenic acid. Highest activity was detected in mycorrhizal roots of nutrient-deprived tomato plants. Surprisingly this caffeoyltransferase activity could also be detected in hydro-alcoholic extracts of *G. intraradices*-colonised roots of leek, sorghum or barrel medic.

1. Introduction

Chlorogenic acid (CGA, 5-caffeoylquinic acid) is one of the most abundant and widespread soluble phenolics in vascular plants [1,2]. Despite indications that it can protect plant cells against oxidative stress [3,4] and that it can play a role in the resistance to phytopathogens [4,5], relatively little is known about its metabolism in plants, apart from the metabolic routes involved in its biosynthesis. This situation contrasts with the numerous animal studies devoted to the absorption and metabolism of CGA, which is an important dietary antioxidant [6–8].

Recent research on CGA metabolism in plants has indeed focused mainly on the elucidation of its biosynthetic pathway [9–14]. cDNA clones encoding the enzyme that directly synthesises CGA from caffeoyl-CoA and quinic acid, *i.e.* hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT), have been characterised from various plants [11–14] and HQT appears to be the principal route of synthesis of CGA in CGA-accumulating plant species such as tomato [4]. It is however known that CGA is not always an end-product of phenylpropanoid metabolism in plants. It is used for example as an acyl donor by chlorogenate-dependant caffeoyltransferase to synthesise various caffeic acid esters [15,16]. It has also been repeatedly reported that it might act as a storage form of caffeic acid and as a precursor of cell-wall bound phenolic polymers [17–19] but so far this hypothesis has not been verified experimentally.

Arbuscular mycorrhizal (AM) fungi, which form symbiotic associations with a wide range of plant species, are a large group of soil microorganisms that play an important role in the mineral nutrition of plants [20] and effectively reduce root disease caused by a number of soilborne pathogens [21]. It has recently been reported that CGA levels significantly decreased in tomato roots upon colonisation by AM fungi [22]. While attempting to measure the CGA content of mycorrhizal tomato roots by HPLC, we noticed that

Abbreviations: AM, arbuscular mycorrhizal; CGA, chlorogenic acid; EDTA, ethylenediamine tetraacetic acid; Fr. wt, fresh weight; HQT, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; LA, Long Ashton; MeOH, methanol; PMSF, phenylmethanesulfonyl fluoride.

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CGA was slowly transformed into an unknown product when the ground roots were extracted in methanol at 6 °C, whereas it was much more stable in non-mycorrhizal root extracts. We eventually established that this transformation of CGA is due to the presence in tomato roots, and especially in mycorrhizal roots, of an enzyme catalysing the transesterification of CGA to methyl caffeate in methanolic extracts. Although the formation of methyl caffeate is an artefact due to the use of methanol during the phenolic extraction procedure, our results show that the transesterification reaction is catalysed by an enzyme exhibiting a distinct substrate preference for CGA. We report here a preliminary characterisation of this enzyme, which could play a role in CGA metabolism, especially in mycorrhizal plant roots.

2. Results

2.1. CGA is slowly converted into methyl caffeate during methanolic extraction of mycorrhizal tomato roots

While measuring the CGA content of *Glomus intraradices*-colonised tomato roots by HPLC, we noticed that it was slowly transformed into an unknown product when the ground roots were extracted for several hours in methanol at 6 °C, whereas it was much more stable in non-mycorrhizal root extracts. The same product was detected when extracting roots harvested 2–6 weeks after inoculation with the fungus. The reaction was also detected in *Glomus mosseae*-colonised tomato root extracts. The newly formed product appeared to derive from CGA since its level gradually increased as the concentration of CGA decreased. The reaction only occurred when the ground roots were left in contact with the hydro-alcoholic extract. Hence no transformation of CGA could be detected in the supernatant obtained after centrifugation of the crude root extracts. By contrast more than 75% of the CGA originally present in mycorrhizal root extracts could be transformed within 2 days at 6 °C in non-centrifuged extracts (Fig. 1). CGA was the major phenolic compound detected at 310 nm in both mycorrhizal and non-mycorrhizal tomato root extracts (Fig. 1A, C and E) and its transformation could therefore be easily monitored by HPLC (Fig. 1B, D and F). The newly formed product was eluted from the reverse phase column later than CGA, was more hydrophobic and could be readily extracted into ethyl acetate. Its UV spectrum was identical to that of CGA, indicating that it could also be a caffeic acid ester (Supplementary data, Fig. 1). When mycorrhizal roots were extracted in ethanol, CGA was transformed in the same conditions, although slightly slower, to a product exhibiting the same UV spectrum (Supplementary data, Fig. 1) but eluting later than methyl caffeate, suggesting that the reaction could be an artefact linked to the use of an alcohol for the extraction (Fig. 1F). This was confirmed by the identification of these two products as methyl and ethyl caffeate, respectively, by co-chromatography with the authentic esters and mass spectrometry (see experimental section). Interestingly the reaction was also detectable in extracts of non-colonised roots but was always 10–25 times slower (Fig. 1B). By contrast it was undetectable in extracts of the aerial parts of tomato plants, which also contain high amounts of CGA, whether or not these plants were inoculated by the AM fungus. As expected, the transesterification of CGA to methyl caffeate (Fig. 2) could also be detected using commercial CGA as substrate. After extraction of endogenous phenolics in MeOH, the root pellet obtained after centrifugation was resuspended in a MeOH–water mixture containing 1 mM CGA. This mixture was then stored at 6 °C and aliquots were analysed at regular time intervals both by HPLC, to monitor the formation of methyl caffeate, and by TLC to monitor the release of quinic acid. Using this approach we found that the transesterification reaction only occurred when the final concentration of water in the methanol–water mixture was *c.a.* 15–35%. With higher concentrations of water rapid oxidation of CGA occurred, leading to the formation of black coloured oxidation products and preventing any methyl caffeate accumulation. By contrast CGA was stable when added to the ground roots in MeOH 100% but the transesterification reaction did not occur. It was also possible to test the effect of pH on the reaction using this procedure, the formation of methyl caffeate being measured in mixtures of buffer and methanol. Maximal formation of methyl caffeate was detected at pH 6, with half activity at pH 7 and pH 4.5. The reaction was easier to detect at 6 °C than at higher temperatures. Although the transesterification took place quicker at room temperature in the first hours of incubation, the final yield of methyl caffeate after 2 days was only *c.a.* 50% of that measured at 6 °C, presumably because of the higher stability of the caffeate esters at this temperature. It was also found that boiling the ground roots in a sealed tube for 5 min in 70% MeOH before adding CGA completely stopped the formation of methyl caffeate. Taken together these results suggested that the transesterification reaction might be an enzyme-catalysed reaction but no definite proof could be obtained at this stage. Although the formation of methyl caffeate was clearly an artefact arising from the use of alcohols for the extraction of phenolics, it seemed interesting to check whether it was really directly linked to the colonisation of roots by the AM fungus and to try to correlate the occurrence of this reaction in mycorrhizal root extracts with a possible change in CGA levels *in planta* [22].

2.2. The rate of transesterification of CGA in tomato root extracts can be modulated by both mycorrhization and mineral fertilisation

It is well established that phosphate inhibits the development of arbuscular mycorrhiza and generally represses genes involved in mycorrhizal functioning [[23], and references therein]. To test whether the rate of the transesterification reaction could be directly linked to the colonisation of tomato roots by the AM fungus, we compared the rate of methyl caffeate formation in crude methanolic extracts of tomato roots grown under contrasting phosphate and mineral supplies. Mycorrhizal and non-mycorrhizal plants were grown for 4 weeks in clay loam soil and watered only with reverse osmosis-purified water, or fertilised 3 times a week with either a modified Long Ashton (LA) nutrient solution with reduced phosphate (LA P/10) or the full strength solution (LA) (Table 1). Four weeks after inoculation the percentage of root length colonised by *G. intraradices* varied from 30 to 2.2% according to the fertilisation mode. As expected in plants watered only with water, growth of both shoots and roots was sharply reduced. A faint positive effect of mycorrhization was detected on the fresh weight of the shoot tissues but only in plants fertilised with reduced phosphate concentration (LA P/10) (Table 1). The different plants were used to measure the CGA levels in the aerial parts (Fig. 3A), in the roots (Fig. 3B), and to compare the rates of CGA transesterification in the methanolic root extracts (Fig. 4). No significant variation in CGA levels could be detected in roots upon mycorrhization 4 weeks after inoculation, whatever the fertilisation mode (Fig. 3B). Mycorrhization induced however an increase in the level of CGA in the aerial parts of plants fed with a nutrient solution (Fig. 3A). This increase was not specific for CGA, since rutin, another abundant phenolic in tomato leaves [24] showed similar variations (data not shown). Fig. 4 shows however that whatever the fertilisation regime, the CGA transesterification rate was always much higher (10–25 fold) in mycorrhizal roots than in controls. Methyl caffeate formation was the most rapid in root extracts of plants watered with water and which exhibited symptoms of mineral nutrient deficiency, in particular reduced growth and CGA