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LOCALIZATION OF INDIVIDUAL β-GLYCOSIDASES IN ALMOND (*Prunus dulcis*) USING A SPECIFIC SUGAR REDUCING ASSAY

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Bitterness in almond (*Prunus dulcis*) is determined by the content of the cyanogenic glucoside amygdalin in the almond embryo. In a previous study (Sanchez-Perez et al., 2008)
Traditional methods to localize total β-glycosidase activity in tissue sections have been based on incubation with the general substrate 6-bromo-2-naphtyl-β-D-glucopyranoside, which when hydrolyzed in the presence of salt zinc compounds gives an in soluble coloured product. This Fast Blue BB method provides a measure of total β-glucosidase activity but cannot distinguish between the localisation of specific β-glucosidases such as amygdalin hydrolase, prunasin hydrolase or any other β-glucosidase. A major difference between sweet and bitter genotypes was observed upon staining of thin sections of tegument and cotyledons for β-glucosidase activity using Fast Blue BB salt with the β-glucosidase substrate 6-bromo-2-naphtyl-β-D-glucopyranoside.
To be able to follow the developmental occurrence of individual β-glycosidases in different almond tissues, we developed a histochemical method that can be used for localization of any β-glycosidase that upon incubation with its specific substrate releases a reducing sugar. Upon hydrolysis of the specific substrate the released sugar is oxidised by a tetrazolium salt (2,3,5-triphenyltetrazolium chloride) that forms a red insoluble product when it is reduced. In Experimental situations where the analyzed tissue has a high content of reducing sugars, a high background staining is observed. This interfering background staining can be avoided if the sections prior to incubation with the specific substrates are incubated with sodium borohydride.
The applicability of the method is demonstrated by tissue and cellular localization of two β-glucosidases - amygdalin hydrolase and prunasin hydrolase - in different tissues and cell types of almond throughout the development of the embryo. The specificity of the devised method is demonstrated in a parallel localization study using a specific antibody towards prunasin hydrolase.

Localisation of β-glucosidases using Fast Blue BB salt

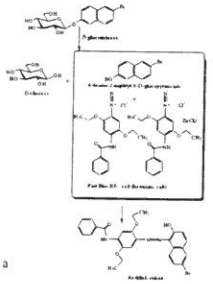

Fig 1a

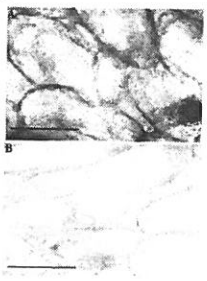

Fig 1b

Several histochemical techniques exist which localize β-glycosidase activity in tissue sections including azo-dies (Cohen, 1952; Ashford, 1970), tetrazonium salt of diorthoanisidine (Rutenburg et al., 1959), or Fast Blue BB salt (Spielman and Mowshowitz, 1981)using mainly 6-bromo-2-naphtyl-β-D-glucoside (BNG) as a general substrate for all β-glycosidases.
In Fig 1a the general reaction scheme for the β-glycosidase reaction using Fast Blue BB salt together with BNG.
Thin almond mesocarp sections (S3067 cultivar, 75 days after flowering (DAF)) was stain with Fast Blue BB salt/BNG to localize total β-glycosidase activity following cleavage of the general substrate BNG a red-brownish stain was produced and indicate the location β-glucosidase activity (Fig 1b panel A).
Background staining from unspecific reaction of Fast Blue BB salt in the absence of added substrate is shown in Fig1b panel B. Bar: 100 µm

Specific localization of β-glucosidase using TTC, reduces glucose released from their specifc substrate

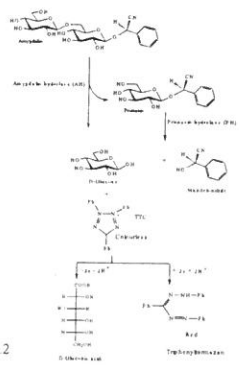

Fig 2

Most β-glucosidases able to hydrolyze CGs show high substrate specificity such as amygdalin hydrolase in *Prunus serotina* (Kuroki and Poulton, 1986) and prunasin hydrolase in *P. serotina* (Kuroki and Poulton, 1987). The two hydrolases release D-glucose from amygdalin and prunasin respectively. This D-glucose can react with TTC to give triphenylformazan which is red. In Fig 2. The reaction scheme fot the detection of amygdalin hydrolase and prunasin hydrolase activity using TTC. The red stain observed in fig3 will be the cells where the hydrolases had been active.
This method was used to localize the amygdalin and prunasin hydrolase throughout the development of the almond embryo.
During development the accumulation of sugars in the embryo tissue became an obstacle as it gave rise to a strong background.

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Reducing background due to the presence of reduces sugars

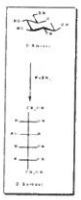

Fig 3a

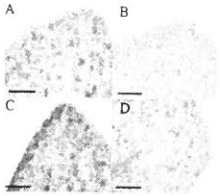

Fig 3b

During the development of the almond embryo the background in sections not treated with the specific substrate grew stronger. The formation of a strong and unspecific background stain due to the presence of reducing sugars was avoidedby prior incubation (5 h or overnight, RT, shaking at 150 rpm) with 50mM sodium borohydride (Fig. 3). The sections of the almond embryo tissue– here cotyledon – was incubated with sodium borohydride as a pre-treatment before amygdalin or prunasin hydrolase localization with TTC. In fig 3 Amygdalin hydrolase was localized in and Kotchkii (88 DAF) (A, B) cotyledons. Samples without substrate were used as controls (C, D). Background was much reduced when cotyledons were pre-incubated with sodium borohydride either for 5 hours (A, C) or overnight (B, D). Bar: 100 µm

Locaization of amygdalin and prunasin hydrolase during development

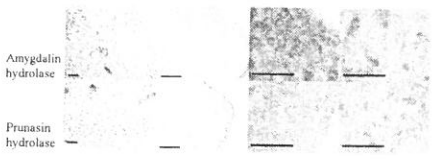

Fig 4 **DAF **DAF **DAF **DAF

During the development of the cotyledon of the almond embryo (here the cultivar S3067) the hydrolase activity is first located in the cells around the vascular tissue with the most prominent stain for purnasin hydrolase. Later the stain accumulates in specific cells where the most abundant stain of glucose released with the respective hydrolases, Amygdalin and prunasin. The stain in these cells are found in the periferi of vesicles in these cells. Bar: 100 µm

Co-localization of amygdalin hydralase be sugar reducing assay and specific anti-bodies

Fig 5

Amygdalin hydrolase is localizes to the chloroplasts in almond mesocarp (S3067 cultivar, 55 DAF) using the sugar reducing assay and amygdalin as substrate (Fig 5 A) whereas no stain is observed when the amygdalng is omitted (Fig 5 B). The same localization to the chloroplast in this tissue is observed when specific antibodies to amygdalin hydrolase is used (Fig. 5 C). Fig 5 D is a light microscopy picture to visualize the green chloroplasts.
Bar: 100 µm

This method enables specific detection of amygdalin or prunasin hydrolyzing β-glucosidases in almond using the specific substrate for each of them. Furthermore it may be used for localization of any β-glycosidase that upon incubation with its specific substrate releases a reducing sugar