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Abstract: In this study the occurrence of a number of enzymes involved in gluconeogenesis was investigated in both tomato fruits and leaves during their development and senescence and in some other fruits. The enzymes studied were phosphoenolpyruvate carboxykinase (PEPCK), pyruvate orthophosphate dikinase (PPDK) and glyoxysomal isocitrate lyase (ICL). PPDK was detected in the ripe flesh of tomato, and much smaller amounts were detected in the flesh of both peach and pepper, whereas it was not detected (not present or at very low abundance) in the other fruits which were investigated (apricot, aubergine, blackberry, blueberry, cherry, grape, plum, raspberry, red current and tomato). By contrast PEPCK was present in the flesh of all the fruits investigated. Very small amounts of ICL were detected in ripe tomato flesh. PEPCK was present in the skin, flesh, locular gel and columella of tomato fruit, and in these its abundance increased greatly during ripening. PPDK showed a similar distribution, however, its abundance did not increase during ripening. PEPCK was not detected in tomato leaves at any stage of their development or senescence. The content of PPDK g-1 fresh weight (FW) increased in tomato leaves as they matured, however, it declined during their senescence. In tomato leaves the content of ICL g-1 FW increased until the mid-stage of development, then decreased as the leaf matured, and then increased during the latter stages of senescence. In the flesh of tomato fruits the contents of PPDK and PEPCK g-1 FW decreased during senescence.
The results suggest that in fruits other than tomato the bulk of any gluconeogeneic flux proceeds via PEPCK, whereas in tomato both PEPCK and PPDK could potentially be utilised. Further, the results indicate that the conversion of pyruvate/acetyl-CoA to malate by the glyoxylate cycle, for which ICL is necessary, is not a major pathway utilised by gluconeogenesis in fruits under normal conditions of growth. Finally, the results contribute to our understanding of the role of several enzymes in the senescence of both leaves and fruits.

Phosphoenolpyruvate carboxykinase, pyruvate orthophosphate dikinase and isocitrate lyase in both tomato fruits and leaves, and in the flesh of peach and some other fruits

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Abbreviations: isocitrate lyase, ICL; malate synthase, MS; malate dehydrogenase, MDH; malic enzyme, ME; oxaloacetate, OAA; phosphoenolpyruvate, PEP; phosphoenolpyruvate carboxylase, PEPC; phosphoenolpyruvate carboxykinase, PEPCK; pyruvate kinase, PK; pyruvate orthophosphate dikinase, PPDK.

Abstract

In this study the occurrence of a number of enzymes involved in gluconeogenesis was investigated in both tomato fruits and leaves during their development and senescence and in some other fruits. The enzymes studied were phosphoenolpyruvate carboxykinase (PEPCK), pyruvate orthophosphate dikinase (PPDK) and glyoxysomal isocitrate lyase (ICL). PPDK was detected in the ripe flesh of tomato, and much smaller amounts

were detected in the flesh of both peach and pepper, whereas it was not detected (not present or at very low abundance) in the other fruits which were investigated (apricot, aubergine, blackberry, blueberry, cherry, grape, plum, raspberry, red current and tomato). By contrast PEPCK was present in the flesh of all the fruits investigated. Very small amounts of ICL were detected in ripe tomato flesh. PEPCK was present in the skin, flesh, locular gel and columella of tomato fruit, and in these its abundance increased greatly during ripening. PPDK showed a similar distribution, however, its abundance did not increase during ripening. PEPCK was not detected in tomato leaves at any stage of their development or senescence. The content of PPDK g^{-1} fresh weight (FW) increased in tomato leaves as they matured, however, it declined during their senescence. In tomato leaves the content of ICL g^{-1} FW increased until the mid-stage of development, then decreased as the leaf matured, and then increased during the latter stages of senescence. In the flesh of tomato fruits the contents of PPDK and PEPCK g^{-1} FW decreased during senescence.

The results suggest that in fruits other than tomato the bulk of any gluconeogeneic flux proceeds via PEPCK, whereas in tomato both PEPCK and PPDK could potentially be utilised. Further, the results indicate that the conversion of pyruvate/acetyl-CoA to malate by the glyoxylate cycle, for which ICL is necessary, is not a major pathway utilised by gluconeogenesis in fruits under normal conditions of growth. Finally, the results contribute to our understanding of the role of several enzymes in the senescence of both leaves and fruits.

Key words: fruits, gluconeogenesis, isocitrate lyase, leaves, malate, peach, phosphoenolpyruvate carboxykinase, pyruvate, pyruvate orthophosphate dikinase, senescence

1. Introduction

Gluconeogenesis from malate occurs in the flesh of ripening grape, tomato and cherry fruits (Farineau and Laval-Martin, 1977; Halinska and Frenkel, 1991; Huang et al., 2015a, 2015b; Leegood and Walker, 1999; Osorio et al., 2013; Ruffner, 1982). In plants gluconeogenesis from malate can occur by two alternate pathways. One pathway

utilises malate dehydrogenase (MDH) in conjunction with phosphoenolpyruvate carboxykinase (PEPCK). The other pathway utilises malic enzyme (ME) in conjunction with pyruvate orthophosphate dikinase (PPDK) (Famiani et al., 2015, 2014b; Leegood and Walker; 2003). In the fruits of both cherry and grape it appears that the PEPCK pathway is used in gluconeogenesis, and this is because PPDK is not present (or is at very low abundance) (Famiani et al., 2014b; Walker et al., 2011a). PEPCK is also present in ripening tomato flesh in which it catalyses a gluconeogenic flux from malate/citrate (Bahrami et al., 2001; Huang et al., 2015a, 2015b; Osorio et al., 2013). In tomato, radiolabelling experiments have shown that gluconeogenesis from pyruvate occurs, and this requires either PPDK or PEPCK (Farineau and Laval-Martin, 1977). If PEPCK is utilised, pyruvate can be converted to malate by either the Krebs cycle or the glyoxylate cycle. Isocitrate lyase (ICL) is an essential component of the glyoxylate cycle (Eastmond and Graham, 2001). In both cucumber flesh and ripening banana flesh very low amounts of ICL are present (Liu et al., 2004; Yang et al., 1998). However, glyoxysomal ICL was not detected in either grape flesh or that of some soft fruits (Famiani et al., 2014b, 2005). In tomato fruits the occurrence of both PPDK and the glyoxylate cycle is uncertain. The first aim of the present study was to investigate the occurrence of PEPCK, PPDK and glyoxysomal ICL in tomato and other fruits. There is contradictory information regarding the occurrence of PPDK in senescing leaves (Chen et al., 2000; Taylor et al., 2010), and very little is known about the occurrence of either PEPCK or PPDK in most fruits during their senescence. The second aim of this study was to determine whether the abundance of PEPCK and PPDK increases in tomato fruits and leaves during their senescence.

2. Materials and methods

2.1. Plant material

Tomato plants (*Solanum lycopersicum*) were grown in a greenhouse in Perugia and fruits were collected at four stages of development. These were: 1, small-green (20% final FW); 2, medium-green (50% final FW); 3, breaker (turning colour); 4, full colouration (red). Plants of sweet pepper (*Capsicum annuum*, cv. World Beater), aubergine (*Solanum melongena*, cv. Black Enorma), *Hoya carnosa* and maize were grown in pots of garden soil in a greenhouse

in Perugia. In 2004, fruits of both peach (*Prunus persica* L. Batsch) cultivar Adriatica and other fruits were collected from mature trees growing in the experimental orchard of the Department of Agricultural, Food and Environmental Sciences of the University of Perugia, in Deruta (PG), central Italy. The fruits of all species were harvested from several positions on the plant. Maize seedlings (*Zea mays*) were germinated and grown in trays of perlite in a greenhouse in Perugia, Italy, and they were then fed NH_4Cl as previously described (Walker et al., 2001). Cucumber cotyledons (*Cucumis sativus*) were obtained from seeds germinated in perlite at 25ºC under darkness for 4 d. For both tomato leaf and fruit senescence experiments mature leaves or ripe fruit (development stage 5) were detached and placed on moist filter paper in petri dishes. These were then placed in an incubator under darkness at 25ºC.

2.2. Preparation of a nitrogen powder

To ensure that the sample was representative of the tissue a nitrogen powder was prepared. Tissues were frozen in liquid nitrogen. When required for analysis, the tissue was removed from the liquid nitrogen. For fruits three samples of the components of the pericarp (skin, flesh, locular gel and placenta), each composed of subsamples of 5-10 fruits, were used. Tissues were then ground together in a mortar containing liquid nitrogen and the resulting powder was used either immediately or after storage at -80ºC. This powder was used for electrophoretic analysis.

2.3. Enzyme assays

Two hundred mg of frozen powder of fruit flesh was ground in a mortar containing 800 µL of ice cold 200 mM Bicine-KOH (pH 9.0), 50 mM DTT and clarified by centrifugation at 12 000 *g* for 5 min. PEPCK activity in the supernatant was measured, using an enzyme coupled method, in the carboxylation direction as described by Walker et al. (1999) and Famiani et al. (2005). For the measurement of PPDK activity plant material was extracted and enzyme activity determined in the forward direction (PEP formation) using an enzyme-coupled spectrophotometric method as described previously (Ashton et al., 1990). For both PEPCK and PPDK one unit of activity is that which produces 1 µmol product min^{-1} at 25 ºC.

2.4. SDS-PAGE and immunoblotting

For pericarp 500 mg of frozen powder was added to 500 μl electrophoresis buffer (62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5% (w/v) SDS, 50 mM ascorbate, 5% (v/v) 2-mercaptoethanol and 0.002% (w/v) bromophenol blue) contained in a mortar, and ground with a pestle. Other tissues were extracted in the same way except that for leaves 100 mg, for roots 250 mg and for either developing or germinating seeds 50 mg of frozen powder was used. If the extract became yellow, several microlitres of 20% (w/v) NaOH were added until it just became blue. The suspension was immediately poured into an Eppendorf tube, which was then incubated at 100 °C for 5 min and then centrifuged at 12 000 *g* for 5 min. The supernatant was separated from the pellet and stored at –20 °C until required. After centrifugation at 10 000 *g* for 5 min, 4 μl of extract was loaded onto each track of SDS-PAGE gels. SDS-PAGE, staining of gels with Coomassie Brilliant Blue dye and immunoblotting were done as described in Walker and Leegood (1996). Protein measurement was done using the Lowry method as previously described (Walker et al., 1995). Briefly SDS-PAGE was performed in a Hoefer mini-gel apparatus (SE 250 Mighty Small II; Hoefer Scientific Instruments, San Francisco, USA) and western transfer done using a Pharmacia Multiphor device (Multiphor II Electrophoresis System; Pharmacia Biotech, Uppsala, Sweden) in conjunction with Millipore Immobilon-P membrane (Millipore, Billerica, Massachutes, USA). Anti-rabbit peroxidase (diluted 1/1000) was used in conjunction with an ECL kit (GE Healthcare, Little Chalfont, UK) to visualize immunoreactive polypeptides. The antisera to aldolase, PEPCK, phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) were produced using as antigens the enzymes that had been purified from *Panicum maximum* leaves (Walker et al., 2002; R.P. Walker, unpublished work). The antisera to PPDK was that used in a previous study of grape (Chastain et al., 2002; Famiani et al., 2014b). ICL antiserum was raised against the enzyme from castor bean endosperm (Maeshima et al., 1988). The glutamine synthetase (GS); antiserum was raised against the plastidic enzyme from *Sinapsis alba* (Höpfner et al., 1990).

3. Results and discussion

3.1. The occurrence of PPDK and PEPCK in the ripe flesh of both tomato and some other fruits

Many studies have established the specificity of both the PEPCK and PPDK antibodies used in the present study for the enzymes from a wide range of plant species (Chastain et al., 2006, 2002; Famiani et al., 2014b, 2005; Taylor et al., 2010; Walker et al., 2011a, 2011b, 2011c). PPDK was detected in both tomato flesh and leaves, and was of the same molecular mass as PPDK from both maize and *Hoya carnosa* leaves (Fig. 1A). PEPCK was detected in tomato flesh, *H. carnosa* leaves, maize leaves but not tomato leaves (Fig. 1A). The occurrence of PEPCK and PPDK in both maize and *H. carnosa* leaves is consistent with previous studies (Black et al., 1996; Leegood and Walker, 1996; Sugiyama, 1973; Walker et al., 1997). The specific activity ([enzyme activity μmol product min^{-1}]/[mg total protein]) of PPDK in tomato leaves is 0.0006 (Hocking and Anderson, 1986) and in maize leaves 0.1-0.19 (Aoyagi and Bassham, 1983; Hocking and Anderson, 1986; Sugiyama, 1973), and these values give an indication of the abundance of PPDK in tomato tissues.

The occurrence of PPDK in the flesh of a number of other fruits was investigated (Fig. 2). All these fruits are known to contain PEPCK (Baldicchi et al., 2015; Famiani et al., 2012, 2005; Walker and Chen, 2002; Walker et al., 2015, 2011a). Under the conditions used for the immunoblot (antibody dilution 1/10 000) PPDK was only detected in tomato (Fig. 2). Similar amounts of PPDK polypeptide were also detected in the ripe flesh of a wide range of tomato cultivars (data not shown). PPDK is also present in the peel of cactus pear fruits, however, in these PPDK functions in Crassulacean acid metabolism (CAM) associated with photosynthesis (Walker et al., 2011c). In keeping with previous work, PPDK polypeptide was not detected in extracts of the ripe flesh of several soft fruits, cherry, plum or grape (Fig. 2; Famiani and Walker, 2009; Famiani et al., 2014b, 2012, 2005; Walker et al., 2011a). To ensure that PPDK polypeptide was not lost after extraction, the flesh of each fruit shown in Fig. 2 was individually co-extracted with one twentieth the FW of maize leaf and then subjected to SDS-PAGE and immunoblotting. In extracts of all fruits co-extracted with maize leaf PPDK was detected, and this shows that PPDK was unlikely to be lost from the fruit tissues (data not shown).

In addition to tomatoes, other solanaceous fruits of commercial importance are aubergines and peppers, and the abundance of both PEPCK and PPDK was determined in their ripening flesh (Fig. 1B). PEPCK was not detected in

sweet pepper, however, it was present in aubergine in which its abundance was very low (Fig. 1B; a barely visible band of the same molecular mass as PEPCK was present in the extract of the flesh of red pepper, this band was so faint that it is not visible in the figure). Previously, very low amounts of PEPCK activity were measured in extracts of aubergine fruits (0.002-0.005 U g^{-1} FW) (Blanke et al., 1988), and this is about 10-fold less than in tomato flesh (Bahrami et al., 2001; Huang et al., 2015a, 2015b; Osorio et al., 2013). PPDK was not detected in aubergine flesh, however, a very long exposure of the immunoblot showed that very low amounts of PPDK were present in the fruit wall of pepper at both stages of development studied (Fig. 1B). However, the band was so faint that it was not possible to reliably determine whether its abundance was different at the two stages of development studied.

We attempted to measure PPDK activity in extracts of the fruits. In peach flesh although PPDK polypeptide has been detected by immunoblotting its activity was not determined (Borsani et al., 2009; Lara et al., 2010, 2009). We failed to measure PPDK activity in the flesh of any of these fruits including tomato (the tissue was harvested and extracted around noon). Previously we failed to measure PPDK in extracts of tomato flesh, and based on this it was stated that there was no evidence for the presence of PPDK in this tissue (Bahrami et al., 2001). To investigate this further we co-extracted ripe fruit flesh with one twentieth its FW of maize leaf (also harvested around noon). This was done for each fruit, and for all co-extractions we could measure a similar amount of PPDK activity as that which was present in the maize leaf extracted alone. Hence it was unlikely that PPDK activity was lost after extraction of the fruit tissues. An explanation for the failure to measure PPDK activity is that in many plant tissues a large proportion of the enzyme is often present as a phosphorylated inactive form (Chastain et al., 2006). For example, in the leaves of the CAM plant Kalanchoë fedtschenkoi PPDK is only active at certain times during the day-night cycle, and for most of the day-night cycle although PPDK protein can be detected its activity cannot be measured (Dever et al., 2015). Therefore, in tomato flesh it is possible that PPDK is often present in an inactive phosphorylated form, and is only active at certain times or under certain conditions.

To have an approximate idea of the abundance of the PPDK polypeptide in tomato flesh different amounts of extracts of either maize leaf or tomato flesh were subjected to SDS-PAGE and immunoblotting. Based on this the amount of PPDK polypeptide mg^{-1} total protein in tomato flesh was about 3-12% of that in maize leaves and on a g^{-1} FW basis

0.2-0.8 % of maize leaves (data not shown). In maize leaves the activity of PPDK is of the order of 1.7 U g^{-1} FW (Aoyagi and Bassham, 1984). Thus the amount of PPDK polypeptide in tomato flesh corresponds to a maximum activity of about 0.003-0.014 U g^{-1} FW. In ripening tomato flesh the activity of PEPCK is about 0.04 U g^{-1} FW (Bahrami et al., 2001; Huang et al., 2015b; Osorio et al., 2013).

In contrast to the present study (Fig. 2), PPDK polypeptide was detected in extracts of ripe peach flesh by immunoblotting, and this was using the same antibody as employed in the present study (Borsani et al., 2009; Lara et al., 2010, 2009). An exposure of a western blot probed with the PPDK antibody at a dilution of 1/10 000 detected a large band of PPDK in maize leaf but nothing in ripe peach flesh (Fig. 3A). By contrast PEPCK was detected in both tissues (Fig. 3A). A western blot was done of extracts of peach flesh (from different stages of its development) and maize leaves (Fig. 3B). An exposure of the western blot, using PPDK antibody at a dilution of 1/10 000, detected a large amount of PPDK in maize leaves but none in peach flesh (Fig. 3B). The blot was reprobed using the PPDK antibody at 1/1000, and a very long exposure showed that PPDK was present throughout the ripening of peach flesh (Fig 5). This shows that in peach flesh PPDK is at low abundance and must be at the very most 10-times less abundant than in tomato flesh. This would give an activity of 0.0003-0.0014 U g^{-1} FW. By contrast PEPCK is quite abundant in peach flesh throughout ripening (about 0.18 U g^{-1} FW; Famiani et al., 2016a). Similar considerations apply to the flesh of cherry, plum and apricot. Thus PEPCK activity in the flesh of these stone fruits is likely over 100-times higher than that of PPDK. Previous studies of both grape and some soft fruits did not detect PPDK in their flesh, and it was concluded that the enzyme was either not present or at low abundance (Famiani et al., 2014b, 2005). The results of the present study suggest that if PPDK is present in the flesh of these fruits, then at most its abundance is similar to that in peach flesh. Thus in the flesh of these PEPCK activity should be at least 35-times higher than that of PPDK. Therefore, as previously suggested in the flesh of all the aforementioned fruits, apart from tomato, the bulk of the gluconeogenic flux should utilise PEPCK and not PPDK. Of course this does not rule out the possibility that in the flesh of these fruits under certain conditions (eg low O_2) the abundance of PPDK increases (Lara et al., 2010).

3.2. The occurrence of PEPCK and PPDK in tomato fruits

PPDK was present at a somewhat similar abundance g^{-1} FW (from stage 1 to stage 4 of development) in the flesh, locular gel and columella of tomato fruits, however, its abundance was lower in the skin of green fruits (Figs. 4, 5A). The abundance of PPDK g^{-1} FW was lower in very young flesh (fruit FW 10% of that of ripe fruit) than in the flesh of fruits later in development (Fig. 5B). Except for in the skin of green fruits, in which its abundance was lower, the abundance of PPDK g^{-1} FW was comparable in the different tissues of both green (developmental stage 2) and ripe tomatoes (developmental stage 4) (Fig. 5A). PEPCK was present in comparable amounts g^{-1} FW in the flesh, locular gel and columella (Fig. 5A). In the skin of green fruits the abundance of PEPCK g^{-1} FW was lower than in the other tissues, and in red fruits slightly higher (Fig. 5A). In the flesh of red fruit the abundance of PEPCK was lower than in the other tissues (Fig. 5A). However, this observation was not representative of the situation because in other experiments the abundance of PEPCK in the flesh was found to be similar g^{-1} FW in the different tissues of the ripe fruit (data not shown). In all the tissues of the fruit the abundance of PEPCK g^{-1} FW increased greatly at the onset of ripening (Fig. 4). In each of these tissues the amount of PEPCK was 0.003 U g^{-1} FW (SE = 0.002) (developmental stages 1,2) and 0.04 U g^{-1} FW (SE = 0.005) (developmental stages 3,4). In blueberry, cherry, plum, grape and peach PEPCK is also present in the different tissues of the fruit (Famiani et al., 2016a, 2012, 2005; Walker et al., 2015, 2011a, 2011b). A previous study also found that there was a large increase in abundance of PEPCK in tomato flesh during ripening, however, it was not detected before ripening in the flesh, or in the locular gel during ripening (Bahrami et al., 2001). It is possible that these contradictions are a result of either difficulties in detecting PEPCK or differences in growth conditions or variety. The presence of PEPCK before ripening is consistent with the presence of PEPCK mRNA in tomato flesh before ripening (Saito et al., 2008; Yin et al., 2010). In several soft fruits, cherry, plum, grape and peach the abundance of PEPCK is also higher during ripening (Famiani and Walker, 2009; Famiani et al., 2016a, 2012, 2005; Walker et al., 2015, 2011a). Nevertheless, in tomato fruits the presence of PEPCK before ripening suggests that the enzyme does not simply function in the dissimilation of malate/citrate during ripening. Ripe tomato fruits were detached from the plant and incubated under darkness at 25ºC for 3-9 d. In the flesh of these fruits the content of both PPDK and PEPCK g^{-1} FW decreased throughout the incubation (Fig. 6).

3.3. PPDK and PEPCK abundance in tomato leaves during their development and senescence

PPDK has been detected in the leaves of some C_3 plants but not in those of others (Bailey and Leegood, 2016; Famiani and Walker, 2009; Hocking and Anderson, 1986; Walker et al., 2011a). In extracts of tomato leaves both PPDK activity and polypeptide have been detected (Famiani et al., 2005; Hocking and Anderson, 1986). PPDK has been found to increase in abundance during the senescence of some leaves, but not in others (Chen et al., 2000; Taylor et al., 2010). We therefore investigated whether PPDK polypeptide content increased in tomato leaves during their senescence. PPDK polypeptide abundance and not PPDK activity was determined, because in the darkened leaves of C_3 plants PPDK is present largely as an inactive phosphorylated form (Chastain et al., 2002). The abundance of PPDK polypeptide mg^{-1} total protein in mature tomato leaves was less than in the fruit (Figs. 7, 8). This comparison was done on a protein basis and not on a FW basis. This was because most of the volume of the cells of the fruit is occupied by a large vacuole and this contains little protein, therefore, the amount of cytoplasm g^{-1} FW (where PPDK, PEPCK are located) in the fruit is less than in the leaf (Famiani et al., 2012). The abundance of PPDK g^{-1} FW increased as the leaf matured (Fig. 8). Similarly the abundance of PPDK increases during the development of both wheat and Arabidopsis leaves (Aoyagi and Bassham, 1984; Taylor et al., 2010). The function of PPDK in mature leaves is uncertain, although as proposed for senescing leaves one function could be in amino acid metabolism (Chastain et al., 2011; Taylor et al., 2010). A preliminary study indicated that the abundance of PPDK g^{-1} FW did not increase in senescing leaves of tomato that were attached to the plant (data not shown), and similarly PPDK g^{-1} FW did not increase in senescing cucumber cotyledons that were attached to the plant (Chen et al., 2000). In tomato leaves the content of PPDK g^{-1} FW declined greatly in leaves detached and incubated under darkness (Fig. 8). Hence in tomato leaves the content of PPDK g^{-1} FW was highest in mature non-senescing leaves. Similarly, when either barley leaves or mature cucumber cotyledons are detached and incubated under darkness, PPDK polypeptide g^{-1} FW declines (Chen et al., 2000). By contrast there is an increase in PPDK abundance mg^{-1} total protein during the senescence of Arabidopsis leaves attached to the plant (Taylor et al., 2010). It is possible that this contradiction is a result of differences between species. An alternative explanation is that in the present study each track on the gel was loaded with the protein content of an equal amount FW of tissue, whereas, in the study of Arabidopsis gels were

loaded with an equal amount of protein (Taylor et al., 2010), and during senescence there is often a large decrease in the total protein content of the tissue (Chen et al., 2000; Fig. 8). Several studies have arrived at the conclusion that PPDK RNA abundance increases during senescence, however, there are potentially difficulties in interpreting these experiments. This is because the decrease in total RNA content of the tissue during senescence was often not considered. It is necessary to show that the abundance of PPDK RNA g^{-1} FW increases: because, as shown by Chen et al. (2000), studies of PEPCK RNA content in senescing tissues which did not do this led to the false conclusion that PEPCK was associated with senescence. Therefore, further experiments to determine whether PPDK protein g^{-1} FW increases during the senescence of leaves from both different species and different tissues would be informative. Nevertheless, these considerations do not affect the proposal that PPDK functions in amino acid metabolism in some senescing leaves (Taylor et al., 2010), and this is because irrespective of whether PPDK g^{-1} FW increases during senescence it is still present.

In tomato leaves, detached from the plant and incubated under darkness, as in barley leaves and cucumber cotyledons (Chen et al., 2000), there was a large decrease in the content of total polypeptides and in the abundance of PEPC and Rubisco (Fig. 8). In tomato leaves there was a large decrease in the larger form of GS (Fig. 8). Two forms of GS were detected and these correspond to the plastidic (larger polypeptide) and cytosolic (smaller polypeptide) GS in tomato (Bortolotti et al., 2003; Fig. 8). In maize root a still smaller form of GS was also present, and this is consistent with previous work which showed that this is a cytosolic GS (Sakakibara et al., 1992). There was a large decrease in the smaller form of aldolase during the senescence of tomato leaves (Fig. 8). A larger form of aldolase was present in maize roots and tomato fruits (Fig. 8). The larger aldolase is the cytosolic enzyme and the smaller one the plastidic enzyme (Famiani and Walker, 2009; Famiani et al., 2014b). Thus the abundance of PEPC, Rubisco, plastidic GS and plastidic aldolase changed in a similar way to that of PPDK (Fig. 8). PEPCK was not detected in tomato leaves at any stage of their development or senescence, and this is consistent with studies of PEPCK RNA content (Fig. 8; Bahrami et al., 2001). Previous studies have shown that PEPCK is unlikely to play a role in leaf senescence (Chen et al., 2000; Taylor et al., 2010). As previously reported (Nieri et al., 1997) small amounts of ICL were present in immature tomato leaves, and in the mature leaf its abundance was much less (Fig. 8). In immature tomato leaves the function of

ICL is unknown (Nieri et al., 1997). In detached leaves of many plants incubated under darkness, there is an induction of ICL and the glyoxylate cycle, and this could potentially function in the anaplerotic replenishment of the Krebs cycle during starvation (Chen et al., 2000; Eastmond and Graham, 2001; Smith, 2002). In detached tomato leaves there was also an induction of ICL (Fig. 8).

3.4. The occurrence of ICL in tomato fruit

In tomato flesh radiolabelling experiments have shown that gluconeogenesis from pyruvate occurs (Farineau and Laval-Martin, 1977). This process can potentially utilise PEPCK in conjunction with the glyoxylate cycle. The enzymes ICL and malate synthase (MS) are essential components of the glyoxylate cycle (Eastmond and Graham, 2001). An early radiolabelling study indicated that MS was present in tomato flesh (Doyle et al., 1960). Both MS RNA and ICL activity are present in the flesh of ripening banana fruits (Liu et al., 2004; Pua et al., 2003). Further, ICL activity has been detected in cucumber flesh (Yang et al., 1998), and in this tissue there is a very low expression of the MS gene (Graham et al., 1992). Glyoxysomal ICL polypeptide and activity were not detected in pumpkin flesh at commercial harvest, however, they subsequently appeared in slices of the flesh incubated under darkness (Pistelli et al., 1996). Glyoxysomal ICL polypeptide was either absent or at very low abundance in the flesh of grape and that of some soft fruits (Famiani et al., 2014b, 2005). In fruits in which ICL activity has been measured it is usually very low: in ripening banana flesh around 0.0001 U g^{-1} FW (Liu et al., 2004) and in cucumber flesh about 0.008 U g^{-1} FW (Yang et al., 1998).

The occurrence of glyoxysomal ICL polypeptide was investigated in tomato flesh using immunoblotting, and the specificity of the antibody employed has been established in previous studies (Chen et al., 2000; Famiani et al., 2014b, 2005). ICL polypeptide was detected in ripe tomato flesh (development stage 4), however, in this it was at a much lower abundance than in the leaf (Fig. 9). Previous studies have shown that ICL polypeptide is present at very low abundance in young tomato leaves (Nieri et al., 1997), and hence it must be at extremely low abundance in tomato flesh. We could not reliably determine how the abundance of ICL polypeptide changed in the different tissues of tomato fruits during their development and senescence, and this was because of difficulties in detecting ICL on

immunoblots. The very low abundance of gyoxysomal ICL in both tomato flesh and that of other fruits, suggests that in these, the conversion of pyruvate/acetyl-CoA to malate by the glyoxylate cycle is not a major pathway utilised by gluconeogenesis under normal conditions of growth. However, recent studies have shown that plants also possess a cytosolic form of ICL with limited sequence similarity to the glyoxysomal form (Eprintsev et al., 2015). This enzyme is thought to function in organic acid interconversions (eg oxalate synthesis in some leaves) and not in the glyoxylate cycle (Eprintsev et al., 2015). Our results do not preclude the presence of this enzyme in fruits, and indeed there is evidence for the occurrence of this enzyme in banana flesh (Eprintsev et al., 2015).

3.5. Gluconeogenesis in tomato fruits

Radiolabelling experiments have shown that gluconeogenesis from malate occurs in tomato flesh (Farineau and Laval Martin, 1977; Halinska and Frenkel, 1991). It is likely that PEPCK is utilised in catalysing this gluconeogenic flux (Bahrami et al. 2001; Huang et al., 2015a, 2015b; Osorio et al., 2013). The bulk of the malate/citrate content of tomato flesh is located in the vacuole (Farineau and Laval-Martin, 1977; Knee and Finger, 1992; Rolin et al., 2000), and this is the most likely source of this malate. Nevertheless, some malate could arise from the metabolism of amino acids and amides, and evidence has been provided that the metabolism of vacuolar malate/citrate could be associated with nitrogen metabolism in the flesh of fruits (Famiani et al., 2016a).

In the flesh of both ripening grape and peach, citrate and/or malate accumulated before ripening can provide only a small amount of substrate utilised by metabolism (Famiani et al., 2016a, 2016b, 2014a). For tomato flesh, by comparing the amounts of CO_2 released from the fruit with the decrease in malate/citrate contents (Biais et al., 2014; Campbell et al., 1990; Chalmers and Rowan, 1971; Knee and Finger 1992), it can be deduced that the situation is similar. A comparison of the amount of glycolytic flux and the amount of CO_2 released from tomato fruits (Campbell et al., 1990; Carrari et al., 2006; Chalmers and Rowan, 1971) indicates that a large proportion of pyruvate so produced is completely oxidised to CO_2 by the Krebs cycle. This is similar to the situation in the ripening flesh of both grape and peach (Famiani et al., 2016a, 2016b, 2014a). Therefore, in tomato flesh glycolysis from sugars is necessary throughout ripening, and this raises the question as to why gluconeogenesis (reversal of glycolysis) occurs.

For both grape and peach flesh one explanation is: the actual rate of malate/citrate dissimilation is not that shown by the changes in their content as determined by two measurements done several days apart. Rather there can be short-term effluxes of malic/citric acid from the vacuole, and this results in a much higher rate of their dissimilation for short periods of time (Famiani et al., 2016a, 2016b; Walker et al., 2015). That is there are times when the amount of malate/citrate released from the vacuole is sufficient to increase the cytosolic malate concentration to a level that brings about gluconeogenesis, and during these periods malate/citrate and not sugars provides the substrate for metabolism (Famiani et al., 2016a, 2016b; Walker et al., 2015). There is evidence to support this proposal in tomato. Firstly, in tomato fruits it appears that there are small diurnal changes in malate content (Farineau and Laval-Martin, 1977). Secondly, reducing the amount of PEPCK activity by around 20% in ripening tomato fruits has an inhibitory effect on gluconeogenesis (Osorio et al., 2013). This is not consistent with the observation that activity of PEPCK is 80-fold higher than the rate of long-term malate dissimilation (i.e. that measured over a period of several days) (Bahrami et al., 2001). However, this is consistent with there being short-term effluxes of malate/citrate from the vacuole, and that during these periods flux through PEPCK is high.

Radiolabelling experiments have shown that gluconeogenesis from pyruvate occurs in tomato flesh (Farineau and Laval Martin, 1977). In the leaves of some CAM plants, PPDK is utilised in gluconeogenesis from pyruvate that is derived from stored vacuolar malate via the action of either cytosolic NADP-ME or mitochondrial NAD-ME (Christopher and Holtum, 1996; Dever et al., 2015; Dittrich, 1976; Häusler et al, 2000). PPDK was present in tomato flesh (Figs. 1A and B, 2, 4), and this raises the possibility that PPDK functions in gluconeogenesis from pyruvate/malate in this tissue. In plants there are both cytosolic and plastidic forms of NADP-ME and a mitochondrial NAD-ME (Famiani et al., 2005). All three enzymes are present in tomato flesh, and their activities appear to be comparable (Bahrami et al., 2001; Jeffery et al., 1986; Knee and Finger, 1992; Knee et al., 1996; Osorio et al., 2013). Tomato flesh contains about 50-times more NAD-ME than does the flesh of either grape or peach (Biais et al., 2014; Borsani et al., 2009; Osorio et al., 2013; Sweetman et al., 2014). The higher abundance of both PPDK and NAD-ME in tomato flesh, compared to that in the flesh of these other fruits, raises the possibility that they can act in concert to catalyse gluconeogenesis from malate/citrate. A recent study showed that pyruvate can be converted

to sugars using the PEPCK pathway in ripening tomato pericarp (Osorio et al., 2013). Hence, it appears that a proportion of pyruvate enters the Krebs cycle and is converted to OAA/malate. A proportion of this OAA/malate then equilibrates with the cytosolic pool of these, and a proportion of this cytosolic pool is then utilised in gluconeogenesis using PEPCK. In plants containing reduced amounts of PEPCK gluconeogenesis from pyruvate was almost abolished (Osorio et al., 2013). However, this does not preclude the utilisation of PPDK in gluconeogenesis because it is possible that PPDK is only used at certain times or under certain conditions. In addition, it is also possible that as in some other tissues pyruvate utilised by PPDK could be generated by amino acid metabolism (Chastain et al., 2011; Taylor et al., 2010). Nevertheless, determining whether PPDK contributes to gluconeogenesis in tomato fruits will require further detailed studies. A scheme depicting the potential pathways utilised in gluconeogenesis in tomato flesh is shown in Fig. 10.

5. Concluding remarks

In the ripe flesh of a range of fruits PPDK polypeptide was only quite abundant in tomato. Smaller amounts of PPDK polypetide were detected in the flesh of both peach and pepper. By contrast PEPCK was present in the flesh of all the fruits investigated. A very small amount of glyoxysomal ICL was detected in ripe tomato flesh. In tomato leaves the content g^{-1} FW of PPDK did not increase during their senescence, and this content was highest when the leaf was mature but not senescent. Both PEPCK and PPDK polypeptides decreased in the flesh of tomato during its senescence. The results suggest that in fruits other than tomato the bulk of any gluconeogeneic flux proceeds via PEPCK, whereas in tomato both PEPCK and PPDK could potentially be utilised. Further, the results indicate that the conversion of pyruvate/acetyl-CoA to malate by the glyoxylate cycle, for which ICL is necessary, is not a major pathway utilised by gluconeogenesis in fruits under normal conditions of growth. Finally, the results contribute to our understanding of the role of several enzymes in the senescence of both leaves and fruits.

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Figure legends

Fig. 1. A) PEPCK and PPDK in ripe tomato flesh and other tissues. Extracts containing the protein content of either 5 mg FW ripe tomato flesh (development stage 4), 0.7 mg FW mature Hoya carnosa leaf, 0.4 mg FW mature tomato leaf or 0.4 mg FW mature maize leaf, were subjected to SDS-PAGE. Polypeptides were then either stained in the gel using Coomassie Brilliant Blue dye or transferred to Immobilon-P membrane and enzymes detected using specific antisera. B) PEPCK and PPDK abundance in solanceous fruits. Extracts containing 5 µg total protein were subjected to SDS-PAGE. Polypeptides were then either stained in the gel using Coomassie Brilliant Blue dye or transferred to Immobilon-P membrane and enzymes detected using specific antisera.

Fig. 2. PPDK abundance in the ripe flesh of tomato (development stage 4) and that of other fruits. Extracts containing the protein content of 4 mg FW of ripe fruit flesh were subjected to SDS-PAGE. Polypeptides were then transferred to Immobilon-P membrane and PPDK detected using a specific antiserum.

Fig. 3. A) Occurrence of PEPCK and PPDK in peach flesh. Extracts containing the protein content of either 5 mg FW ripe peach flesh or 0.4 mg FW mature maize leaf, were subjected to SDS-PAGE. Polypeptides were then transferred to Immobilon-P membrane and PPDK detected using a specific antiserum. The dilution of PPDK antiserum was 1/10 000. B) Occurrence of PEPCK and PPDK in peach flesh. Extracts of either 5 mg of peach flesh (from peach fruits at different stages of development; these are the same extracts as used in Famiani et al. 2016a) or 0.4 mg FW mature maize leaf were were subjected to SDS-PAGE. Polypeptides were then transferred to Immobilon-P membrane and PPDK detected using a specific antiserum. The dilution of PPDK antiserum was 1/10 000 (short exposure) and 1/1000 (long exposure).

Fig. 4. PEPCK and PPDK abundance in the flesh, locular gel or columella of tomato during development. Extracts containing the protein content of either 5 mg FW tissue (flesh and columella) or 15 mg FW tissue (locular gel) were

subjected to SDS-PAGE. Polypeptides were then transferred to Immobilon-P membrane and enzymes detected using specific antisera. The developmental stage of the fruits was based on their size and/or colour: 1, small-green; 2, medium-green; 3, half colouration; 4, full colouration.

Fig. 5. A) PEPCK and PPDK abundance in different parts of tomatoes (green fruits = development stage 2, red fruits = developmental stage 4). Extracts containing 5 µg total protein were subjected to SDS-PAGE. Polypeptides were then transferred to Immobilon-P membrane and enzymes detected using specific antisera. B) PEPCK and PPDK in the flesh of either very young tomato fruit (fruit FW 10% of that of ripe fruit) or ripe fruit (developmental stage 4). Extracts containing the protein content of 5 mg FW were subjected to SDS-PAGE. Polypeptides were then transferred to Immobilon-P membrane and detected using specific antisera.

Fig. 6. PEPCK and PPDK abundance in ripe tomato pericarp post-harvest. Tomato fruits were detached from the plant and incubated under darkness at 25ºC for either 3, 6 or 9 days. Extracts corresponding to 4 mg FW of tissue were subjected to SDS-PAGE. Polypeptides were then either stained in the gel using Coomassie Brilliant Blue dye or transferred to Immobilon-P membrane and enzymes detected using specific antisera.

Fig. 7. The abundance of polypeptides in tomato leaves, at different stages of development and senescence, and in other tissues. Extracts corresponding to either 1 mg FW of leaf or 4 mg FW of either fruit or root were subjected to SDS-PAGE. Polypeptides were then stained in the gel using Coomassie Brilliant Blue dye.

Fig. 8. The abundance of PEPCK, PPDK, ICL and other enzymes in tomato leaves, at different stages of development and senescence, and in other tissues. Extracts corresponding to either 1 mg FW of leaf or 4 mg FW of either fruit or root were subjected to SDS-PAGE. Polypeptides were then transferred to Immobilon-P membrane and enzymes detected using specific antisera.

Fig. 9. ICL abundance in young tomato leaves and the flesh of ripe tomato fruits (development stage 4). Extracts containing 5 µg total protein were subjected to SDS-PAGE. Polypeptides were then transferred to Immobilon-P membrane and ICL detected using a specific antiserum.

Fig. 10. Simplified metabolic scheme showing potential pathways utilised by gluconeogenesis in tomato flesh. NAD-ME = NAD-malic enzyme, NADP-ME = cytosolic NADP-malic enzyme, OAA = oxaloacetate, PEP = phosphoenolpyruvate, PEPCK = phosphoenolpyruvate carboxykinase, PPDK = pyruvate, orthophosphate dikinase. Note that the bulk of the malate and citrate in the vacuole is almost certainly synthesised from sugars. Nevertheless a small proportion could arise from the carbon skeletons of amino acids and amides.

Figure 1

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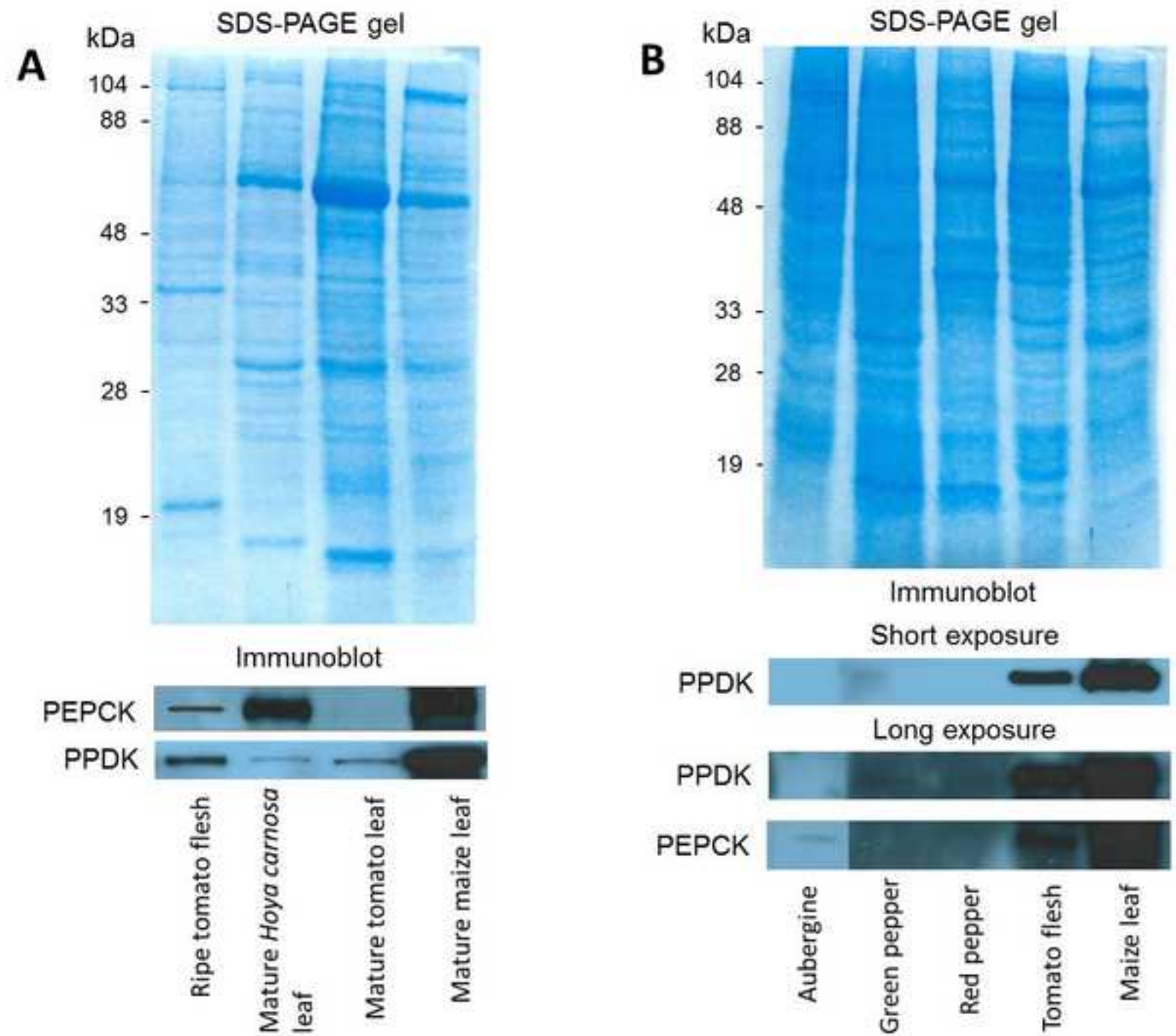

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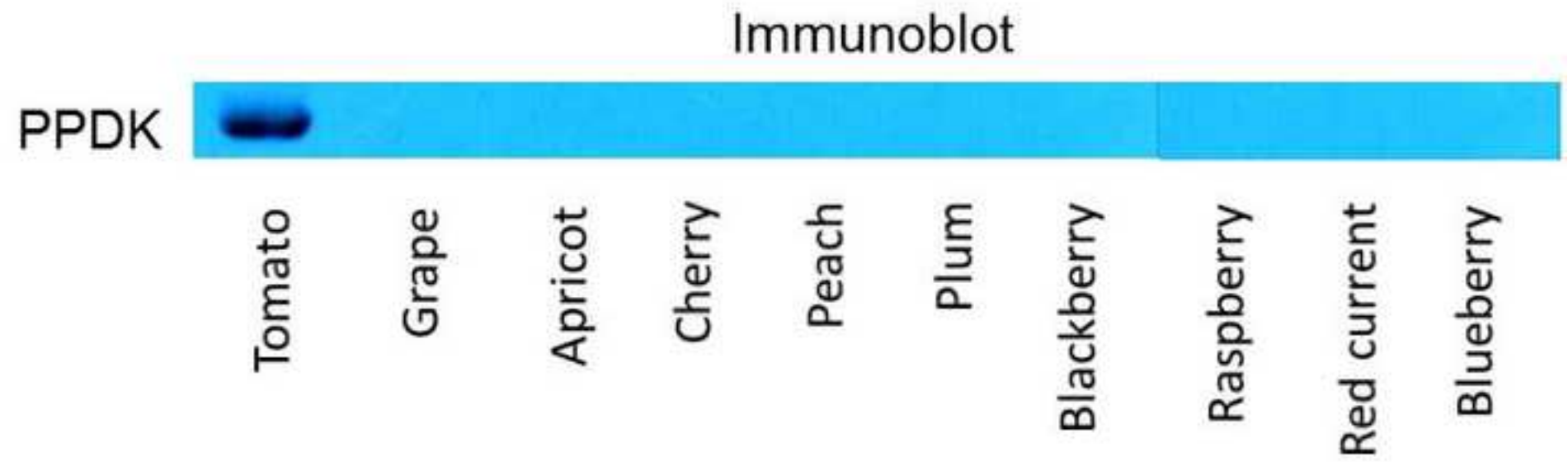

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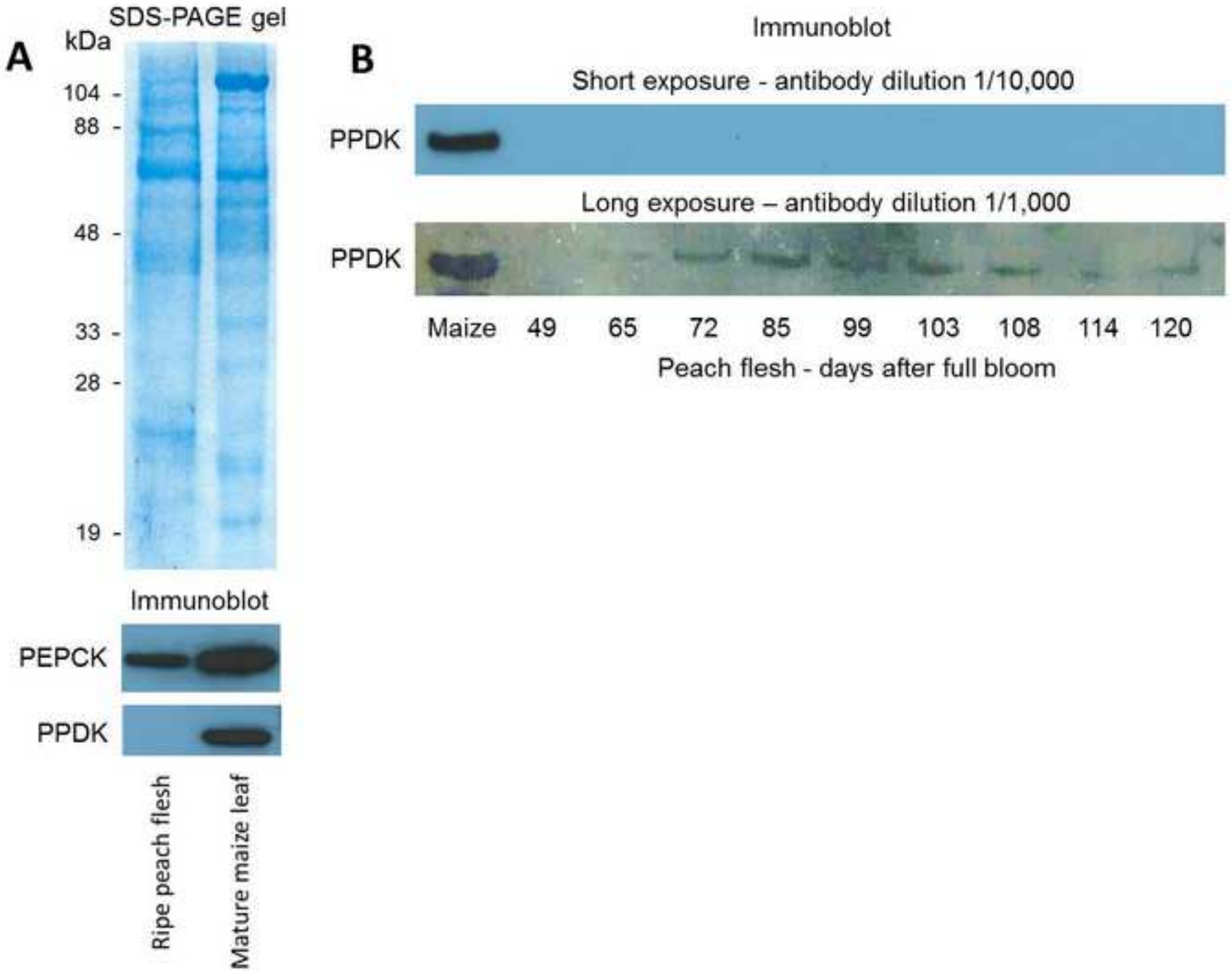

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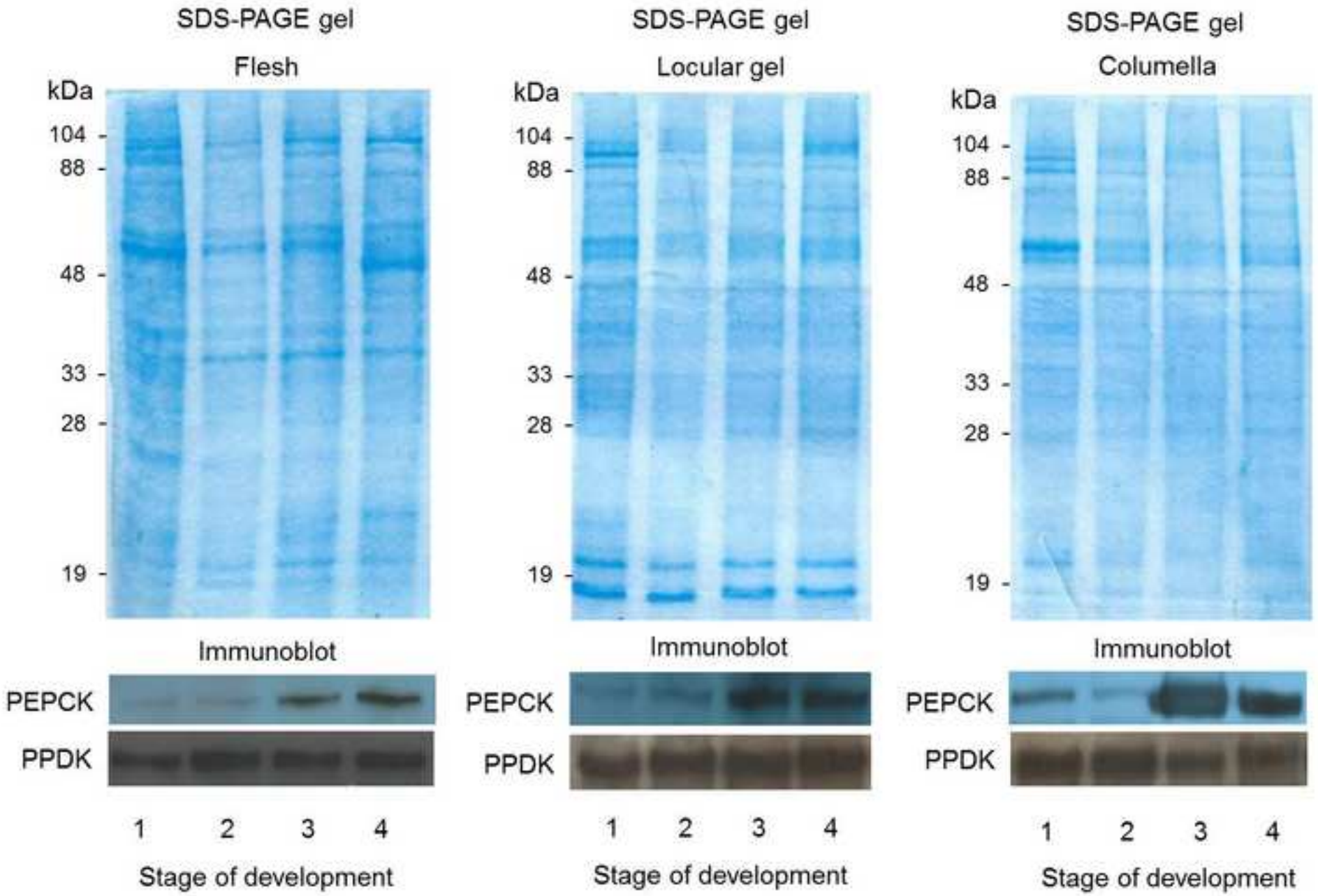

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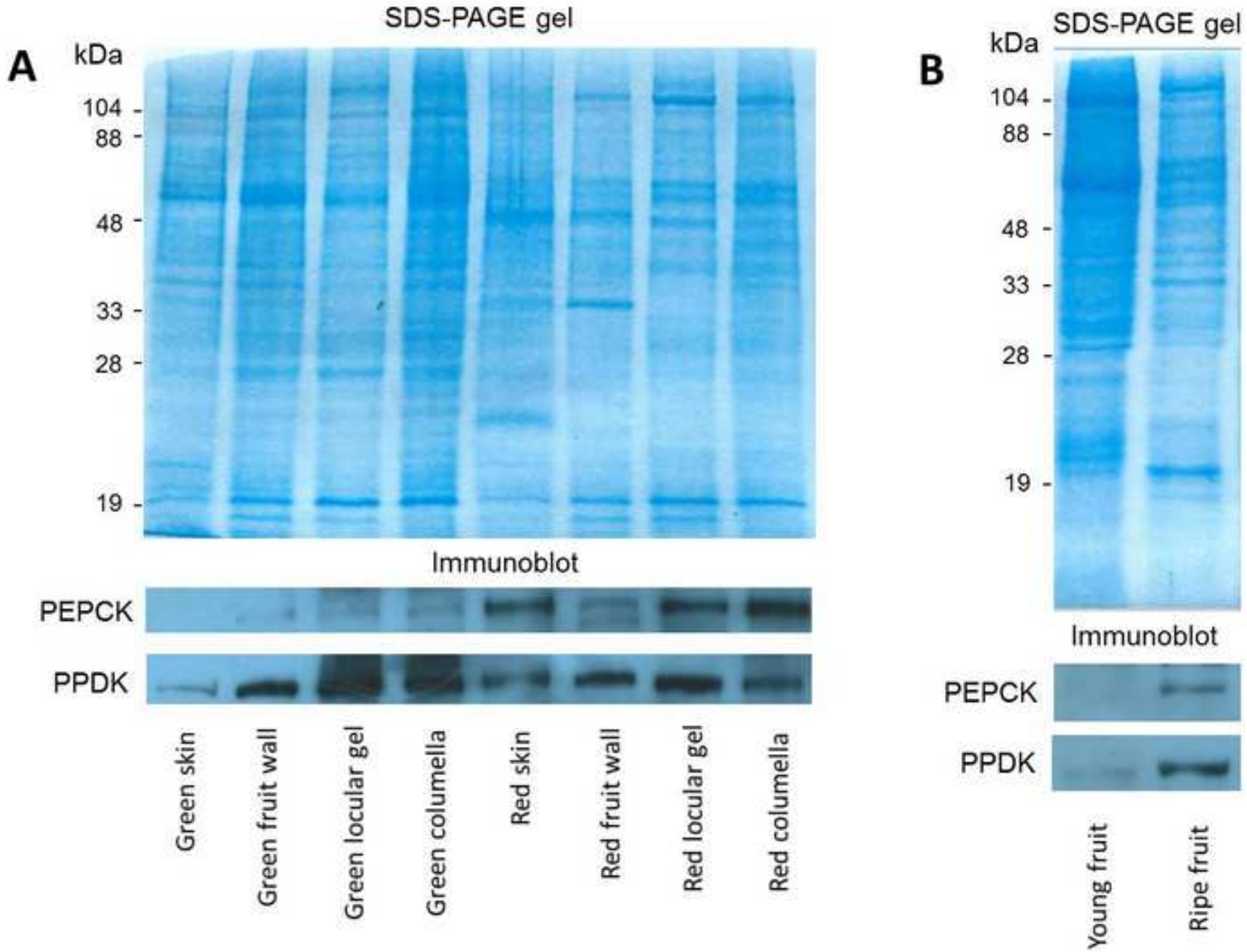

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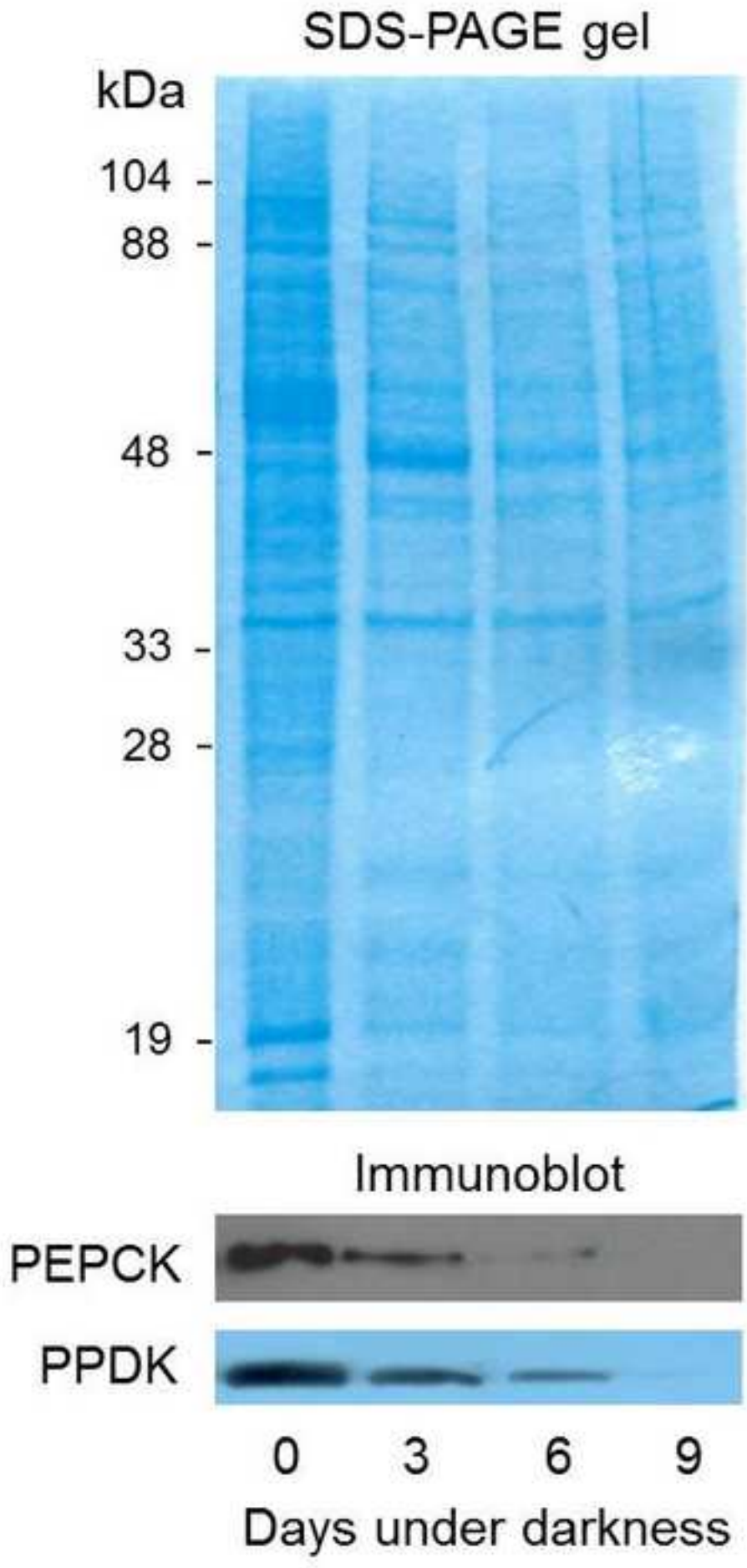

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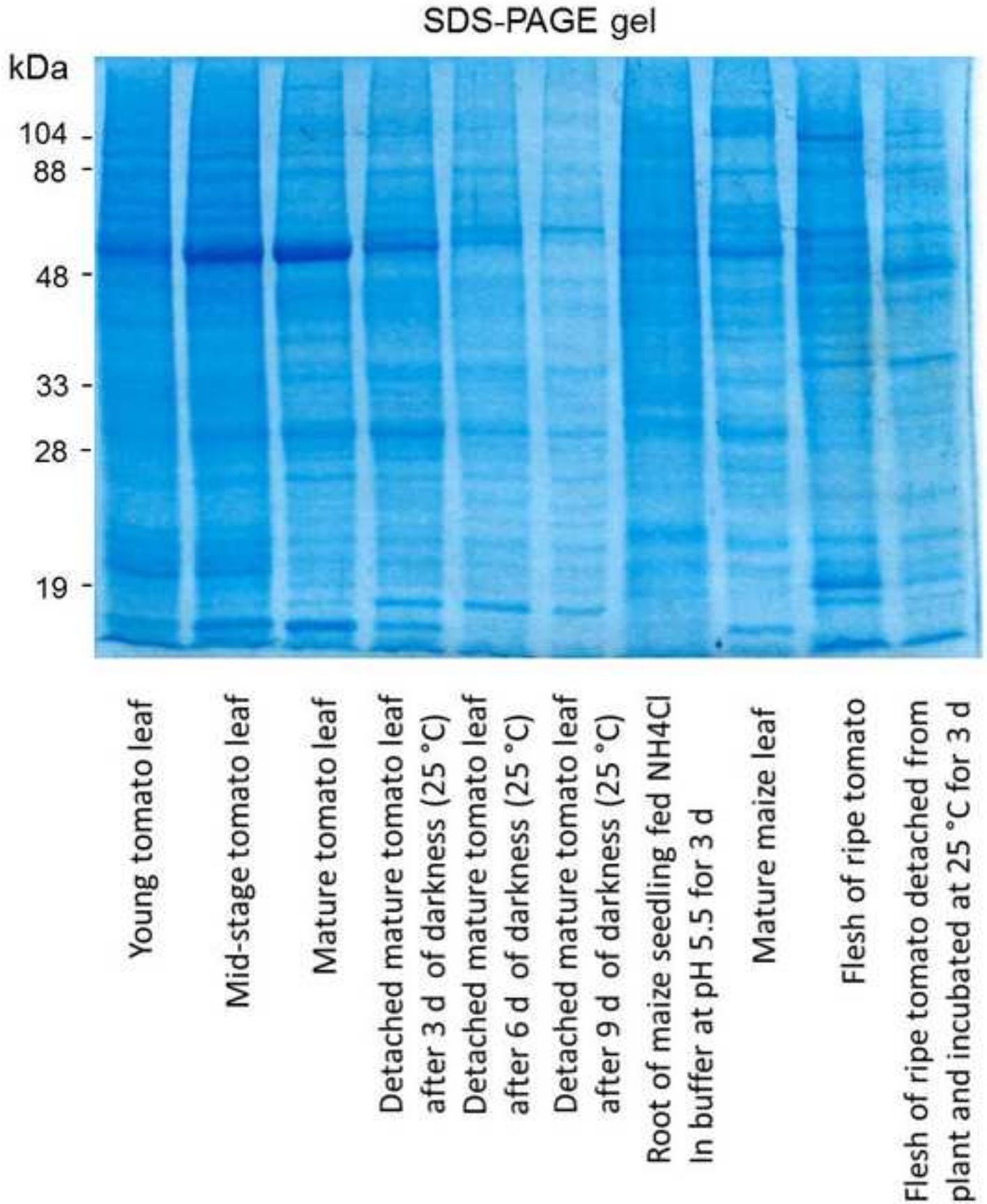

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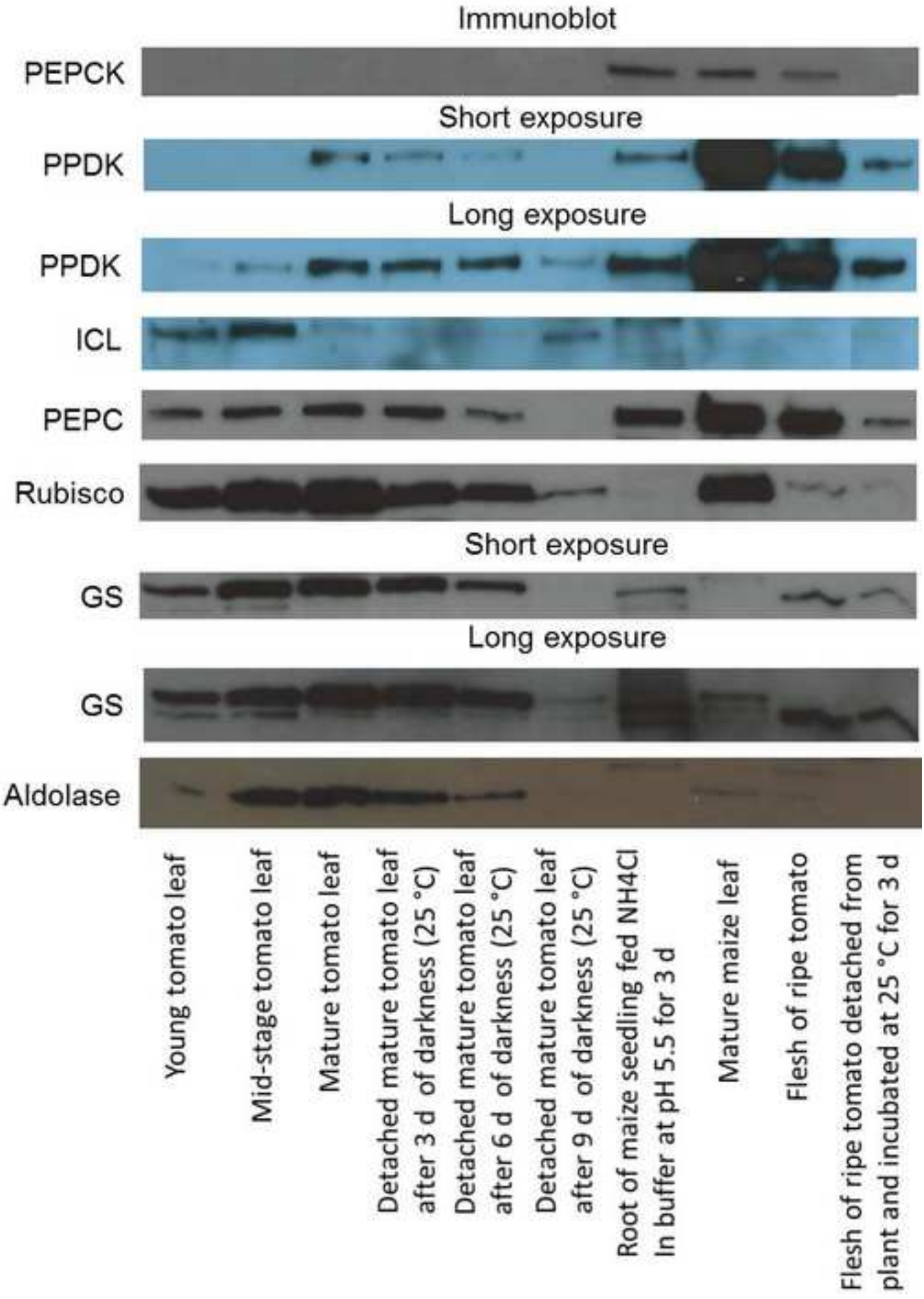

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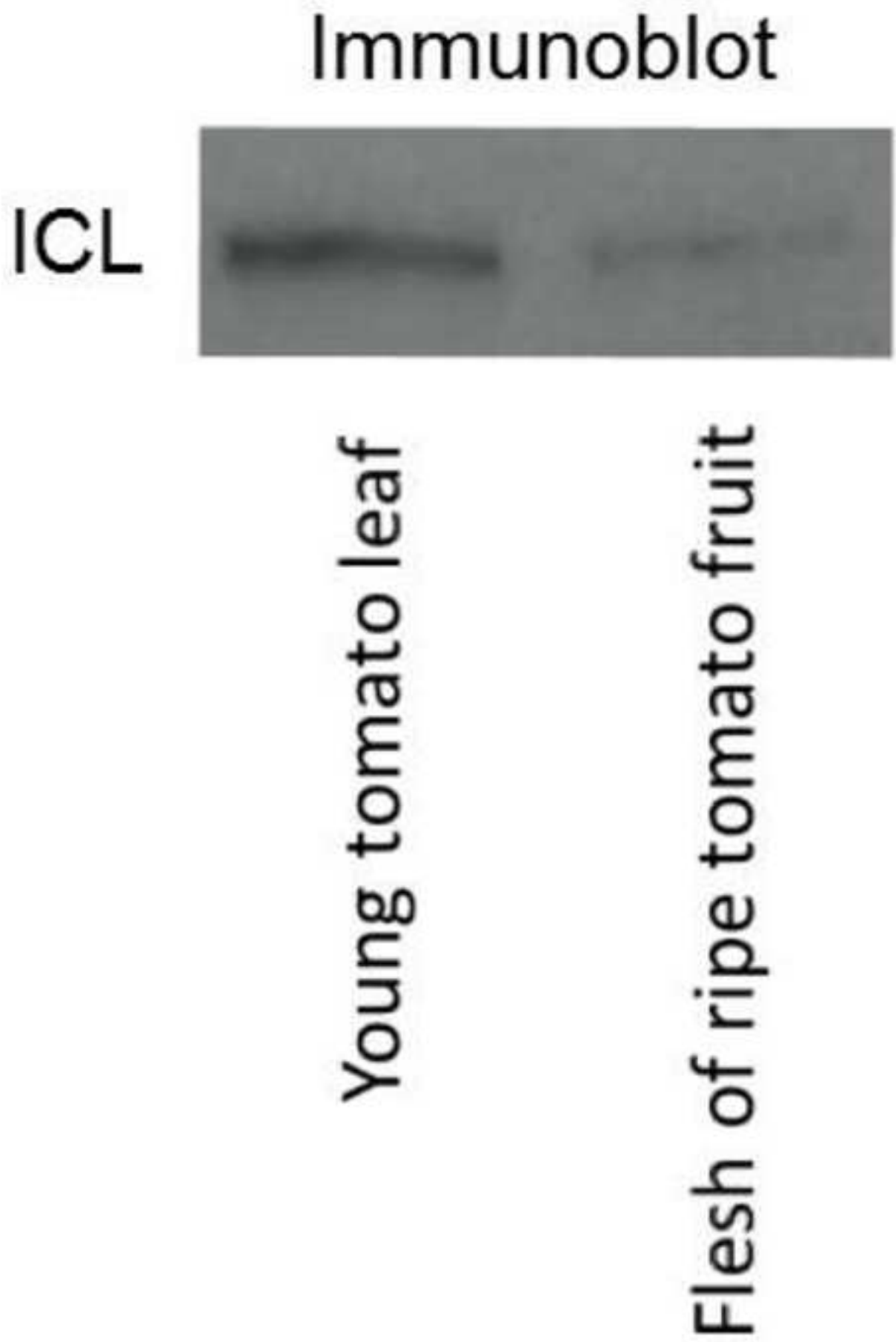

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