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ORIGINAL ARTICLE

Nitrogen-deficiency-induced loss in photosynthesis and modulation of β-galactosidase activity during senescence of *Arabidopsis* leaves

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Abstract Senescence-induced loss in the content of chlorophyll and the rate of oxygen evolution is remarkably enhanced when the leaves of *Arabidopsis thaliana* experience nitrogen-deficiency stress. On the other hand, the decline in the level of total soluble sugar during senescence is very slow and nitrogen deficiency does not exhibit any further change. The relative stability in the level of the sugar in the background of severe decline of photosynthesis may suggest the contribution of sugars from other sources to sustain its homeostasis to execute and complete energy-dependent senescence process and stress response. The possible participation of cell wall polysaccharides contributing to sugar homeostasis is predicted. Senescence-induced increase in the activity of β-galactosidase (EC 3.2.1.23) and its further enhancement in senescing leaves experiencing nitrogen stress support the proposition of participation of the enzyme for breakdown of the wall polysaccharides to sugars. The loss of photosynthesis as a possible signal for enhancement in the activity of β-galactosidase has been further examined in the excised leaves incubated in Okada and Shimura (OS) nutrient medium with and without nitrogen. Nitrogen limitation experienced by excised leaves causes rapid loss in photosynthesis with concomitant increase in the activity of the enzyme extracted both from soluble and cell wall fractions. The differential activity of the enzyme from soluble and cell wall fractions during development-dependent leaf senescence and premature senescence in excised leaves induced by nitrogen deficiency appears to be complex and needs to be resolved in the future.

Keywords Photosynthesis · Senescence · β-Galactosidase · Nitrogen deficiency

Introduction

Leaf senescence plays a key role in nutrient recycling and thus significantly modulates plant growth, development, and reproduction (Hörtensteiner and Feller 2002; Biswal et al. 2003, 2013; Lim et al. 2007; Guiboileau et al. 2010; Thomas 2013). In spite of accumulation of large volume of data, the nature of the mechanism, induction, and regulation of the process is not fully understood. The process follows a pattern of events with temporal expression of several genes with some genes expressed early and some late (Fujiki et al. 2001, 2005; Gepstein et al. 2003; Breeze et al. 2011). Currently, we have some understanding of the signaling systems, including sugar signaling associated with the pattern of time-dependent expression of senescence-associated genes (*SAGs*) (Guo and Gan 2005; Fischer 2012). It is important to note that execution and completion of senescence program are energy dependent but ironically the process of senescence in green leaves is well known to bring down the rate of photosynthesis, consequently loss of the production of sugars, the major source of energy (Grover 1993; Biswal et al. 2003; Keskitalo et al. 2005; Thomas 2013). In this scenario, some of the genes upregulated during senescence participate in a cellular catabolic network to provide respiratory sugar to energy starved cells by the degradation of cellular constituents, including polysaccharides, proteins and lipids

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(Biswal et al. 2003; Lin and Wu 2004; Buchanan-Wollaston et al. 2005; Troncoso-Ponce et al. 2013). On the other hand, sugars, in addition to their metabolic role to provide energy, are also reported to act as signaling molecules for induction and regulation of leaf senescence (Yoshida 2003; Gibson 2005; van Doorn 2008; Wingler and Roitsch 2008; Biswal and Pandey 2016). The possible role of sugars for regulation of expression of *SAGs* and activity of senescence-associated enzymes is known (Yoshida 2003; Baena-González et al. 2007; Lee et al. 2007; Mohapatra et al. 2010). The late expression of a *SAG* (*din2*) that codes for cell wall bound β-glucosidase and enhanced activity of this enzyme during the late phase of senescence is suggestive of possible participation of cell wall polysaccharides to sustain sugar homeostasis during terminal phase of senescence (Fujiki et al. 2005; Mohapatra et al. 2010; Patro et al. 2014). Since cell wall largely remains intact with, however, some structural modifications during the terminal phase of senescence (Biswal et al. 2003), the wall polysaccharides appear to be the last target for senescence related catabolism.

Abiotic stress modulates plant senescence (Sedigheh et al. 2011; Khanna-Chopra 2012; Patro et al. 2014; Chen et al. 2015). However, the exact nature of developmentally regulated senescence and the process induced by stress still remains unresolved (Buchanan-Wollaston et al. 2005; Carp and Gepstein 2007). Nitrogen deficiency is one of the abiotic stresses that are well reported to cause loss of photosynthesis and result in premature senescence (Schulte auf'm Erley et al. 2007; Schildhauer et al. 2008; Balazadeh et al. 2014). Carbon-to-nitrogen (C/N) ratio is a key factor that determines growth and development including senescence (Martin et al. 2002; Aoyama et al. 2014). It is also well known that assimilation of nitrogen is largely dependent on photosynthetic production of sugar. Because of crucial role of nitrogen in sugar metabolism, its deficiency is monitored in laboratory condition to examine its interaction with senescence and its possible modulation of sugar level. We have demonstrated that deficiency of nitrogen causes remarkable decline in photosynthesis during senescence and modulates the level of total soluble sugar possibly through different metabolic routes. In the background of severe loss in photosynthetic production of sugar, the activity of β-galactosidase (EC 3.2.1.23), a cell wall bound enzyme, has been examined and the question of degradation of cell wall polysaccharides, richest source of organic carbon in the plant body to cellular sugar pool, has been addressed. There are several glycoside hydrolases that are known to participate in the degradation of cell wall polysaccharides (Iglesias et al. 2006; Minic and Jouanin 2006). *Arabidopsis thaliana* genome codes for 17 β-galactosidase genes of glycosyl hydrolase family 35 (Ahn et al. 2007; Chandrasekar and van der Hoorn 2016). β-

Galactosidase participates in the degradation of cell wall polysaccharides as well reported during fruit ripening and in the cotyledons of germinating seeds (Edwards et al. 1988; Ranwala et al. 1992; Ali et al. 1995; Kitagawa et al. 1995; Balasubramaniam et al. 2005). The enzyme is also reported to breakdown cell wall polysaccharides in *A. thaliana* during stress response (Contento et al. 2004; Lee et al. 2004, 2007). The physiological behaviour of this enzyme has not been examined so far during leaf senescence, although the upregulation of expression of the gene coding for α-galactosidase and the activity of the enzyme are reported earlier (Chrost et al. 2004, 2007). We have examined the response of the β-galactosidase extracted both from soluble and cell wall fractions of the senescing leaves of *A. thaliana* to nitrogen deficient environment.

Materials and methods

Plant material and growth conditions

The plants of *A. thaliana* ecotype col-0 were grown in culture room that maintains a temperature of 23 ± 2 °C. The seedlings were grown in pots on a soil mixture of perlite, vermiculite, and sphagnum moss in a ratio of 1:1:1 with long photoperiod (18 h light/6 h dark). The seeds were surface sterilized with 2% (v/v) sodium hypochlorite with a drop of Triton-X 100 for 5 min. The pots for the growth of plants were soaked overnight with Okada and Shimura (OS) medium (Okada and Shimura 1990), and seeds were kept at 4 °C for 48 h and then transferred to plant culture room. Plants were grown in light flux of 90 µmol m^{-2} s^{-1}. The leaves from the first rosette of 20–35-day-old *Arabidopsis* plants experiencing senescence phase (Mohapatra et al. 2010) were used for all the experiments.

To examine the response of senescing leaves to nitrogen-limiting environment, growth of the seedlings in OS medium was monitored as per the following:

The seedlings were grown in the standard OS medium with the addition of 100 ml of the medium during germination and subsequently with 30 ml on every third day until the 35th day of the experimental period. One set was grown in the OS medium with recommended concentration of nitrogen salt [5.0 mM KNO$_3$, and 2.0 mM Ca(NO$_3$)$_2$] (non-limiting, Control), and the second set was grown in the OS medium for the first 6 days after germination followed by the medium without any addition of nitrogen salt (nitrogen-limiting). In this set, the nitrogen salts were replaced by chloride salts, as Ca (NO$_3$)$_2$ replaced by CaCl$_2$ and KNO$_3$ replaced by KCl (Schildhauer et al. 2008; Balazadeh et al. 2014). The second set was referred as the plants grown in nitrogen limiting or nitrogen deficit condition.

The leaves from the first whorls of *Arabidopsis* plants grown in nitrogen-limiting condition were excised after 20 days of germination and floated in Petri dish containing OS medium with and without nitrogen salts for 72 h.

Chlorophyll quantification

Ten mg leaves of *Arabidopsis* plants were crushed in 100% acetone. The mixture was centrifuged at 2500g at room temperature, and the extract was used for estimating the chlorophyll content. The pigment content was determined according to the method of Lichtenthaler (1987) using Varian Cary 50 Bio UV–Visible spectrophotometer.

Soluble sugar estimation

The soluble sugar content was estimated by the method of Buysse and Merckx (1993). 25 mg of fresh leaf materials was homogenized in 5 ml of 80% alcohol and kept in water bath for 1 h at 60 °C. After centrifugation at 2500g for 15 min, 0.5 ml leaf extract was transferred to a thick walled test tube and 0.5 ml of 18% phenol was added to it and mixed thoroughly. 2.5 ml of concentrated sulfuric acid ($\sim$98% pure) was then added to it immediately and mixed thoroughly by vertical agitation with a glass rod. Absorbance was recorded at 490 nm. The corresponding concentration was determined against a standard curve prepared using the glucose solution.

Measurement of oxygen evolution

Photosynthetic oxygen evolution of leaf samples was measured as described by Mohapatra et al. (2010). The samples were placed in the leaf chamber unit of leaf disc electrode (LD2/3, Hansatech instruments) and irradiated from the red light-emitting diode (LED) source (LH 36/2R, Hansatech instruments) with an intensity of 1000 µmol m^{-2} s^{-1}. The rate of oxygen evolution was measured as a function of incident photon flux density (Mohapatra et al. 2010).

Extraction of soluble and cell wall bound fractions of β-galactosidase from the leaves of *Arabidopsis* plants with different treatments

The leaves ($\sim$0.5 g) collected from plants with different treatments were homogenized with the buffer containing 100 mM sodium citrate (pH 4.6) and 10 mM β-mercaptoethanol in a chilled mortar and pestle (Kitagawa et al. 1995). The homogenate was centrifuged for 15 min at 2500g at 4 °C. The precipitate was extracted for two more times with the above buffer, and all the supernatants were pooled for buffer soluble fraction. The precipitate was stirred for 1 h with the buffer containing 100 mM sodium citrate (pH 4.6), 13 mM EDTA, 10 mM β-mercaptoethanol, 2% (w/v) soluble polyvinylpyrrolidone (PVP-40), and 1 M NaCl. After stirring, the homogenate was centrifuged for 35 min at 12000g at 4 °C. The precipitate was extracted two more times with the above buffer and all supernatants were taken as cell wall fraction for assay of enzyme activity as per the procedure of Balasubramaniam et al. (2005). For extraction of total homogenate for the enzyme activity, the same buffer and procedure were adapted as used for extraction of cell wall bound enzyme fraction.

Enzyme assay of β-galactosidase

The enzyme activity for β-galactosidase (EC 3.2.1.23) was determined by the procedure described by Balasubramaniam et al. (2005) by measuring the change of absorbance at 415 nm. The assay mixture with 100 mM sodium citrate (pH 4.1) and 13 mM *p*-nitrophenyl-β-D-galactopyranoside as substrate was taken and the enzyme extract was incubated at 37 °C for 30 min. One unit of the enzyme activity was defined as the amount of β-galactosidase releasing 1 µmol of *p*-nitrophenol per minute in the reaction mixture under the assay conditions. The reaction was terminated by adding 2 ml of 0.2 M NaOH. The activity was measured by taking *p*-nitrophenol as the standard.

Protein estimation of the enzyme extract

The protein content of the enzyme extract was estimated as per Lowry et al. (1951), using bovine serum albumin as standard.

Statistical analysis

Statistical analysis was performed using the SPSS 22 Microsoft compatible software. The results presented are mean ± standard deviation of three replicates ($n = 3$). Duncan test was applied for mean separation to show a significant difference among the treatments at $P < 0.05$ significance level.
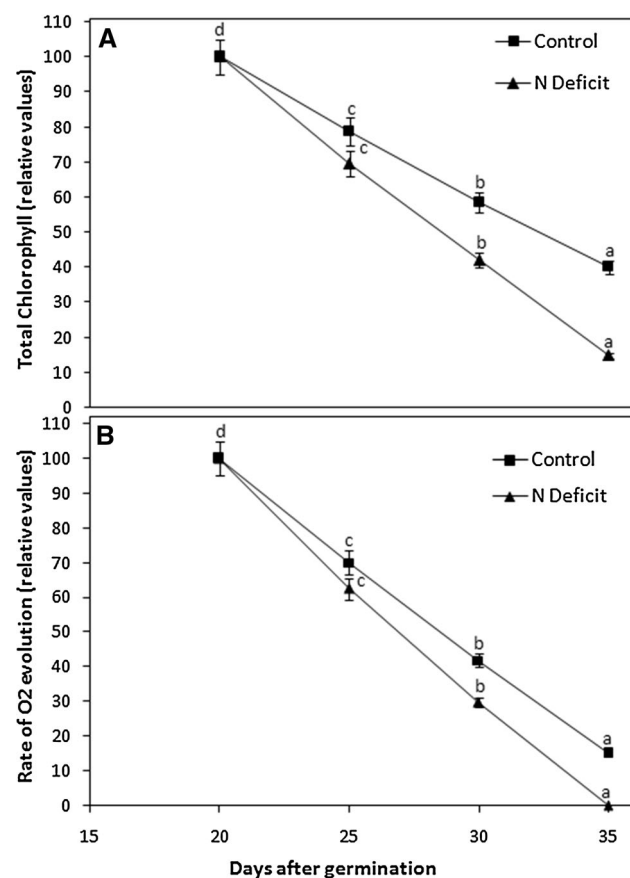
Results

Nitrogen deficiency induced changes in the contents of chlorophyll, rate of oxygen evolution, total soluble sugar, and in the activity of β-galactosidase of intact leaves during senescence.

Changes in the content of chlorophyll

Nitrogen-limiting environment shows relatively more loss in the content of the pigment during senescence (Fig. 1a). On the 35th day, the losses in the pigment content were 61% in the senescing leaves (control) and 85% in the senescing leaves experiencing nitrogen deficit environment (N deficit).

Decline in the rate of oxygen evolution

The decline of photosynthetic oxygen evolution during senescence was enhanced by nitrogen deficiency (Fig. 1b). The loss of oxygen evolution on the 35th day was found to
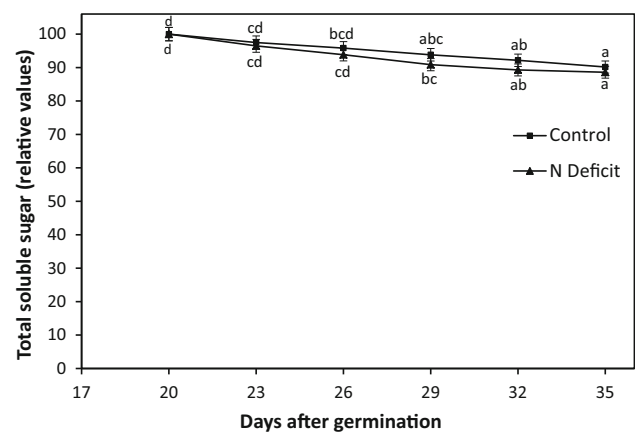
be 85% in senescing samples compared to its complete loss in senescing leaves experiencing nitrogen-limiting conditions.

## Changes in total soluble sugar

There is a gradual and slow decline in the levels of total soluble sugar in senescing leaves and the leaves experiencing nitrogen limitation during 20–35 days of senescence period (Fig. 2). It is important to note that the loss of sugar level both during senescence and senescing leaves experiencing nitrogen stress was not very significant compared to the losses in the content of the pigment and rate of oxygen evolution. The loss during senescence on the 35th day was only 10%, and it was 11% in the senescing leaves experiencing nitrogen stress.

Nitrogen limitation enhances the activity of β-galactosidase during senescence

The changes in the activity of β-galactosidase extracted from total homogenate during senescence period from the 20th to 35th days are shown in Fig. 3. The activity of the enzyme was significantly enhanced in partially senescent leaves measured on the 29th day (S1), and it was further enhanced on the 35th day (S2), and the increase in the activity was 178% over the enzyme activity in mature leaves on the 20th day. The changes in the activity of β-galactosidase extracted both from soluble and cell wall

Fig. 1 Changes in the relative values of **a** chlorophyll content and **b** rate of oxygen evolution of *A. thaliana* leaves during senescence for a period of 20–35 days of the plants grown in OS medium with recommended nitrogen salts (control) and nitrogen-limiting condition (N deficit). The values obtained on the quantity of chlorophyll content and rate of oxygen evolution of mature leaves measured on the 20th day after germination are normalized to 100%. Absolute values of chlorophyll content and rate of oxygen evolution on the 20th day samples for control and N deficit conditions are **a** 1.23 and 1.15 mg g^{-1} fr wt^{-1} and **b** 9.02 and 6.34 µmol m^{-2} s^{-1}, respectively. The *error bars* represent ± SD ($n = 3$). *Bars followed by different letters* show a significant difference at $P < 0.05$ significance level according to Duncan test

Fig. 2 Changes in the relative values of total soluble sugar of *A. thaliana* leaves during senescence for a period of 20–35 days of the plants grown in OS medium with recommended nitrogen salts (control) and nitrogen-limiting condition (N deficit). The values obtained on the quantity of total soluble sugar of mature leaves measured on the 20th day after germination are normalized to 100%. Absolute values of the 20th day samples for control and N deficit conditions are 6.0 and 5.7 µg mg^{-1} fr wt^{-1}, respectively. The *error bars* represent ± SD ($n = 3$). *Bars followed by different letters* show a significant difference at $P < 0.05$ significance level according to Duncan test
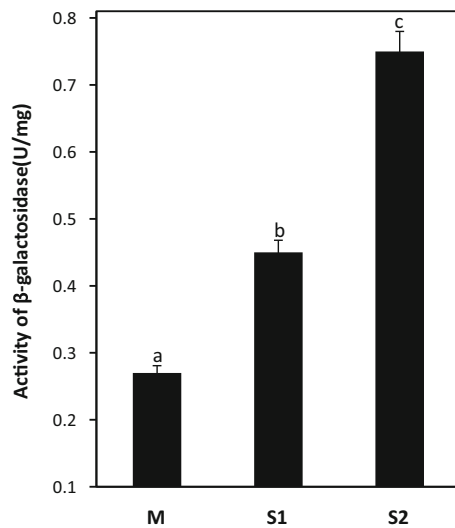
Fig. 3 Changes in the activity of β-galactosidase of *A. thaliana* leaves during senescence for a period of 20–35 days of the plants grown in OS medium with recommended nitrogen salts. The activity of β-galactosidase was measured on the 20th day (mature leaves; *M*), 29th day (partially senescent leaves; *S1*), and 35th day (senescent leaves; *S2*) after germination. One unit (U) enzyme activity = amount of enzyme releasing 1 μmol of product per minute. The *error bars* represent ± SD ($n = 3$). *Bars followed by different letters* show a significant difference at $P < 0.05$ significance level according to Duncan test
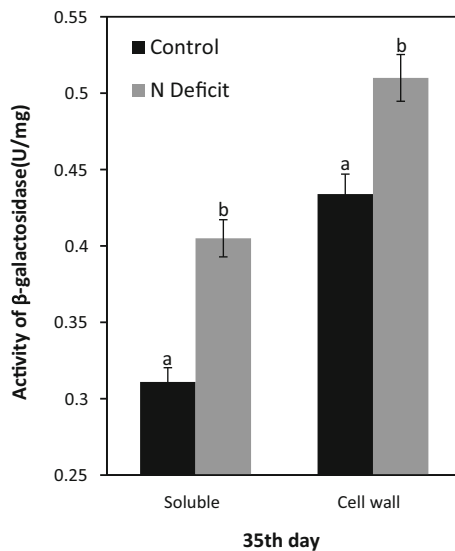
Fig. 4 Activity of β-galactosidase in soluble and cell wall fractions of *A. thaliana* leaves measured on the 35th day of germination (senescent leaves) of the plants grown in OS medium with recommended nitrogen salts (control) and nitrogen-limiting condition (N deficit). One unit enzyme activity (U) = amount of enzyme releasing 1 μmol of product per minute. The *error bars* represent ± SD ($n = 3$). *Bars followed by different letters* show a significant difference at $P < 0.05$ significance level according to Duncan test

fractions on the 35th day of senescing leaves, and senescing leaves experiencing nitrogen deficit stress are shown in Fig. 4. The activity of β-galactosidase both from soluble
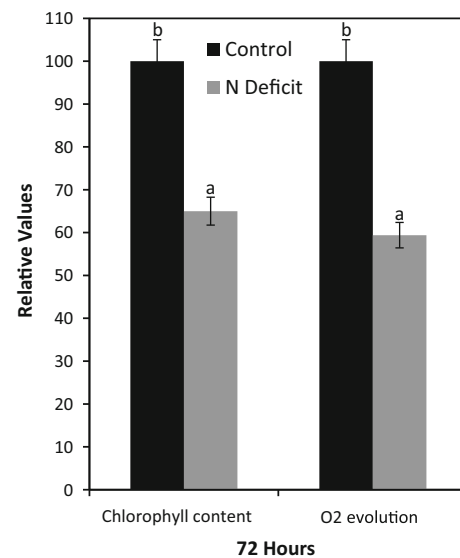
Fig. 5 Relative values of chlorophyll content and rate of oxygen evolution of fully mature excised *A. thaliana* leaves floated in OS medium with recommended nitrogen salts (control) and OS medium without nitrogen salts (N deficit) for 72 h in continuous white light (90 μmol m^{-2} s^{-1}). The values of quantity of chlorophyll content and rate of oxygen evolution measured for control samples are normalized to 100%. The absolute values of total chlorophyll content and oxygen evolution for control samples are 1.2 mg g^{-1} fr wt^{-1} and 8.3 μmol m^{-2} s^{-1}, respectively. *Vertical bars* represent ± SD ($n = 3$). *Bars followed by different letters* show a significant difference at $P < 0.05$ significance level according to Duncan test

and cell wall fractions in nitrogen-limiting condition was enhanced significantly.

The changes in the content of chlorophyll, rate of oxygen evolution, and the activity of β-galactosidase in excised mature leaves of 20-day-old *A. thaliana* incubated with the standard OS medium with and without nitrogen for 72 h.

To further examine the response of β-galactosidase to nitrogen-limiting condition, the leaves excised from the 20-day-old plants are floated in OS medium with and without nitrogen addition. Nitrogen deficiency causes a remarkable loss in the content of chlorophyll and rate of oxygen evolution measured after 72 h of incubation (Fig. 5). Not unexpected, the activity of the enzyme extracted both from soluble and cell wall fractions is significantly suppressed in the leaves floated in full strength OS medium over the leaves floated in OS medium without the addition of the nitrogen salts (Fig. 6).

Discussion

Senescence-induced decline in photosynthesis and its further decline with senescing leaves experiencing nitrogen depletion are not unexpected. The literature on the loss of photosynthesis during leaf senescence and abiotic stress
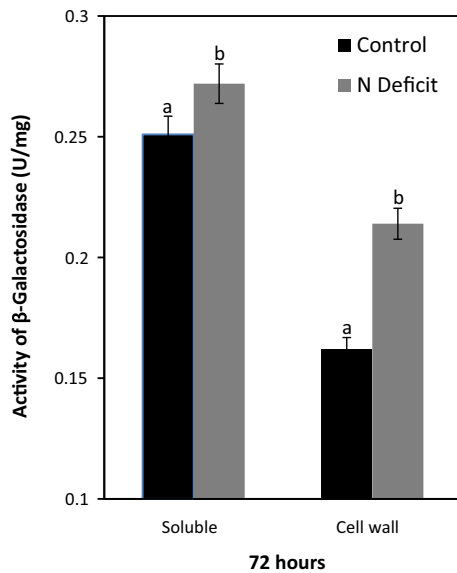
Fig. 6 Activity of β-galactosidase of soluble and cell wall fractions of fully mature excised *A. thaliana* leaves floated in OS medium with recommended nitrogen salts (control) and OS medium without nitrogen salts (N deficit) for 72 h in continuous white light (90 μmol m^{-2} s^{-1}). One unit (U) enzyme activity = amount of enzyme releasing 1 μmol of product per minute. *Vertical bars* represent ± SD ($n = 3$). *Bars followed by different letters* show a significant difference at $P < 0.05$ significance level according to Duncan test

response is rich (Noodén et al. 1997; Chaves et al. 2009; Ashraf and Harris 2013; Biswal and Pandey 2016). However, relatively a remarkable stability of total soluble sugars (Fig. 2) in the background of severe loss of photosynthesis (Fig. 1) makes an important point in the present work. Nitrogen deficiency does not bring any significant change in the level of sugars compared to the changes observed during senescence (Fig. 2). Nitrogen plays a key role in regulating the endogenous pool of sugars. Its assimilation potential and availability modulate consumption of sugars. The relative stability of sugar (Fig. 2) in spite of rapid loss of photosynthesis during senescence and in nitrogen depletion environment may be attributed to supply of sugars from the other metabolic route(s) without direct involvement of photosynthesis. Remobilization of nitrogen from senescing leaves to upper growing plant parts (Biswal et al. 2003; Masclaux-Daubresse et al. 2008; Thomas 2013; Avila-Ospina et al. 2014) and poor availability of nitrogen in leaves during growth of the plants in nitrogen-limiting environment may cause reduction in the level of cellular nitrogen that is likely to result in apparent increase in the accumulation of sugars (Balazadeh et al. 2014). Senescence- and stress-induced activation of catabolism for degradation of cellular lipids, proteins and polysaccharides (Buchanan-Wollaston 1997; Biswal et al. 2003; Yoshida 2003; Buchanan-Wollaston et al. 2005;

Troncoso-Ponce et al. 2013; Avila-Ospina et al. 2014) to sugar may be considered as one of the routes that could contribute to the sustenance of sugar level in spite of rapid loss in photosynthesis.

Sugar is believed to act as a signaling molecule regulating plant senescence (Yoshida 2003; Baena-González et al. 2007; Biswal et al. 2012; Biswal and Pandey 2016). It is also a source of cellular metabolic energy. The sustenance of sugar homeostasis in the background of rapid loss of photosynthesis, as shown in Fig. 1, may be considered as an adaptational strategy of the leaves to execute and complete energy-dependent senescence program and stress response. The activity of β-galactosidase both during senescence and senescing leaves experiencing nitrogen deficit stress was studied in the present work to examine the possible participation of the hydrolase that breaks down cell wall polysaccharides and contributes to total sugar pool. Rapid decline in photosynthesis parallels the senescence-induced enhancement in the activity of the enzyme (Fig. 3) with further enhancement in its activity in the senescing leaves experiencing nitrogen limitation, as shown in Fig. 4. Similar observation on the loss of photosynthesis as a possible factor for enhancement in the activity of β-glucosidase, another cell wall bound hydrolase during senescence and drought stress response has been earlier made in *Arabidopsis* (Mohapatra et al. 2010; Patro et al. 2014). These observations are suggestive of loss of photosynthesis as a possible signal for activation of the enzyme which may participate in the breakdown of cell wall polysaccharides. The gene expression and catalytic activity of β-galactosidase bound to the cell wall in *Arabidopsis* has been examined earlier (Perez Almeida 2004). The photosynthetic modulation of the enzyme activity is further confirmed in the excised leaves floated in the OS medium with and without nitrogen for 72 h (Fig. 6). The activity of β-galactosidase is enhanced (Fig. 6) with concomitant loss in photosynthesis as characterized by the content of chlorophyll and the rate of oxygen evolution in the leaves floated in OS medium without nitrogen (Fig. 5). It is important to note that the activity of the enzyme extracted from cell wall fraction is much higher than its activity from soluble fraction of intact senescing leaves of the plants grown with and without nitrogen-deficiency environments (Fig. 4) in contrast to relatively much less activity of the enzyme in the wall fraction extracted from the excised leaves incubated for 72 h (Fig. 6). This differential responses of the cell wall bound enzyme activity of intact senescing and excised leaves to nitrogen deficit stress may suggest operation of differential regulatory mechanisms in development-dependent senescence and premature senescence induced by the abiotic stress. It is logical to argue that the cell wall polysaccharides may be the target of the wall bound enzymes and the increase in its

activity, therefore, is not unexpected. Relative increase in the activity of the enzyme in soluble fraction during premature senescence induced by abiotic stress is difficult to explain, and it is not certain if the enzyme in soluble fraction plays a role in the degradation of cell wall polysaccharides and thus participates in sugar reprogramming in the background of senescence-induced loss in photosynthetic production of sugars.

Author contribution statement The research work was carried out in the laboratory of BB. All the authors have equal contribution in this work. JKP and SKD did all the experiments. JKP and BB analyzed the results and wrote the paper.

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