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Influence of ultraviolet-C on the compositions of cell-wall polysaccharides and carbohydrase activities of *Silene vulgaris* callus

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Abstract—UV-C irradiation (254 nm) was found to enhance the secretion of some cell-wall-degrading enzymes, especially the following carbohydrases: β-galactosidase, α-L-arabinofuranosidase, polygalacturonase, pectinesterase, cellulase, xylanase, and β-xylosidase, in the campion callus, contributing thereby to an alteration in the polysaccharide structure. The relative amounts of the galactose and arabinose residues in pectin (silenan) and of arabinose in arabinogalactan of calli irradiated during the exponential phase were shown to decrease during the stationary phase. A decrease in the degree of SV methylesterification was found for the irradiated callus. These alterations were found to persist over a long period of culturing time. Decreasing the relative amounts of the arabinose residues in arabinogalactan and pectin and the galactose residues in silenan corresponded to increasing activity of α-L-arabinofuranosidase and β-galactosidase, respectively, due to treatment with UV-C. UV-C irradiation may be used as a tool for modifying the structural features of the cell-wall polysaccharides, such as the relative amounts of galactose and arabinose residues in the side chains of polysaccharides, with the purpose of obtaining physiologically active polysaccharides with the desired properties and structural features.

Keywords: *Silene vulgaris*; Cell cultures; Ultraviolet-C; Carbohydrases; Pectin

1. Introduction

The preliminary studies of *Silene vulgaris* (M.) G. (*Oberna behen* (L.) I.) callus tissue indicated the synthesis of polysaccharides possessing immunomodulatory activity.[1] The *S. vulgaris* callus appeared to be an alternative to the plant as the source of this valuable product. The polysaccharides of the campion callus have been shown to include pectin (named silenan) and acidic arabinogalactan.[2] The structural features of silenan and arabinogalactan have been elucidated earlier.[3,4] Silenan samples from the intact plant and from the callus were shown to contain the linear α-(1→4)-D-galacturonan backbone with 2-substituted α-L-rhamnopyranose residues and ramified regions. The silenan side chains were composed of blocks containing the terminal and α-(1→5)-linked D-arabinofuranose, β-(1→3)-linked, and β-(1→4)-linked D-galactopyranose residues.[3,4] The core of the arabinogalactan consisted of different segments of β-(1→3)-D-galactopyranan. The side chains of the arabinogalactan were shown to contain the residues of terminal and 3-O-substituted β-D-galactopyranose, terminal α-D-arabinofuranose and α-D-rhamnopyranose, and 2-O-substituted α-L-rhamnopyranose.[4] The quantitative and qualitative variations in the compositions of polysaccharides during the growth cycle of a campion culture, as well as the influence of hormonal factors and the effects of sugar, calcium, phosphate, and nitrogen on cell growth and polysaccharide production, have been studied earlier.[2,5–8]

Metabolic activity in relation to the cell wall is known to be modulated in response to stress, and changes in the wall's composition due to salt or other osmotic factors and pathogens have been documented.[9] The other origin

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of stress is connected with the response to UV-C. Barka et al.[10] have earlier suggested that the cell-wall-degrading enzymes are one of the targets of UV-C, and by this action, irradiation contributed to a delay in degradation of the cell wall and consequently retarded the softening of tomato fruit tissues. A diversity of responses, depending on the species studied, nature of the UV treatment and interaction with other environmental factors, has been observed.[11] Morphogenetic changes in plants depend on the sensitivity of the species and their inherent ability to attenuate the incoming UV radiation.[11] The deleterious effects of UV radiation are known to include damage of DNA, peroxidation of lipids, photo-oxidation of pigments, and inhibition of photosynthesis.[11–15] The induction of a number of defense mechanisms, such as the production of UV-B screening pigments, increasing amounts of antioxidant enzymes, and the induction of pathogenesis-related proteins, is mediated at the level of gene expression.[14,15] A wide range of plant defense responses to UV radiation was investigated; however, little is known about the mechanisms involved.

The effects of UV-C on the compositions of cell-wall polysaccharides and carbohydrase activities in plants have not been investigated previously. The present work appears to be useful for the elucidation of plant responses to UV-C stress. The new data allow us to use this factor as a possible means of modifying polysaccharides to obtain physiologically active polysaccharides with the desired properties and structural features.

The present paper is devoted to studies of UV-C effects on the compositions of cell-wall polysaccharides and on carbohydrase activities in the campion callus.

2. Results and discussion

2.1. Cell-wall polysaccharide compositions in the exponential growth phase 24 h after irradiation with UV-C

Campion callus tissue was irradiated with UV-C (254 nm) in the exponential growth phase (on day 12) for 10–60 min, followed by analysis 24 h after irradiation.

Alteration of the exposure duration over a range of 0–60 min failed to influence the yields, volumetric production per liter of medium, and polysaccharide compositions of silenan SV and arabinogalactan AG 24 h after irradiation. The yields of SV and AG were found to be 4.7–6.9% and 5.8–8.0%, respectively. The volumetric production per liter of medium was calculated to be 0.24–0.36 and 0.28–0.43 g L^{-1} for SV and AG, respectively. The percentages of both galacturonic acid (50–66%) and neutral monosaccharides in silenan were close, relative to the control data (unirradiated cells). The residues of galactose (3.1–4.3%) and arabinose (3.0–3.9) were shown to be the main neutral sugars of SV. Rhamnose, xylose, mannose, and glucose residues were also detectable. Qualitatively and quantitatively, the sugar compositions of acidic arabinogalactan were found to be similar in irradiated and unirradiated cells. The main neutral sugars in this fraction were galactose (41.5–44.2%) and arabinose (12.1–15.9%), with negligible amounts of rhamnose, xylose, mannose, and glucose. The ratios of galactose/arabinose in arabinogalactan were 3.1 and 2.8–3.4 in unirradiated and irradiated cells, respectively.

Thus, irradiation by UV-C in the exponential growth phase (12th day) failed to have an influence on the production and sugar composition of both silenan and arabinogalactan 24 h after irradiation. These data demonstrated that incubation for 24 h after irradiation was insufficient for the development of changes in the biochemical characteristics of the cell-wall polysaccharides. For this reason, callus tissue irradiated during the exponential growth phase (12th day) was analyzed in the stationary phase (21st day).

2.2. Effect of UV-C on the compositions of cell-wall polysaccharides and carbohydrase activities in the stationary phase

Changing the duration of exposure in the range from 10 to 60 min failed to influence the production of polysaccharides in comparison with the control data (unirradiated cells) but affected the sugar composition both of silenan and arabinogalactan. The yields of SV and AG were found to be 5.2–7.2% and 5.4–6.6%, respectively. The volumetric production per liter of medium was shown to be 0.35–0.47 and 0.36–0.42 g L^{-1} for SV and AG, respectively.

The percentages of galacturonic acid (70–81%) in SV were close to the control data (78%). The amounts of residues of galactose and arabinose in SV were shown to decrease during the stationary phase independently of the exposure duration (Fig. 1). The galactose/arabinose ratio in SV was found to be unchanged in comparison with the control (1.3–1.6). The amounts of arabinose residues in AG from irradiated callus tissue decreased 1.4–1.9-fold independently of the irradiation duration. The galactose/arabinose ratio in AG was found to increase in comparison with the control from 4.3 to 5.7–7.3. The degree of SV methylesterification was found to be 6.9–8.1% for the unirradiated callus, while methylesterification decreased to 1.5–3.8% in SV of the irradiated cells.

The molecular weight distributions of SV and AG were measured using ultrafiltration through membranes. Fraction SV-I with an M_w greater than 300 kDa was found to be dominant (yield was 75%) in unirradiated cells. Fractions SV-II with an M_w of 100–300 kDa (yield was 2%), and SV-III with an M_w of 50–100 kDa (yield was 0.4%) were shown to be minor fractions in

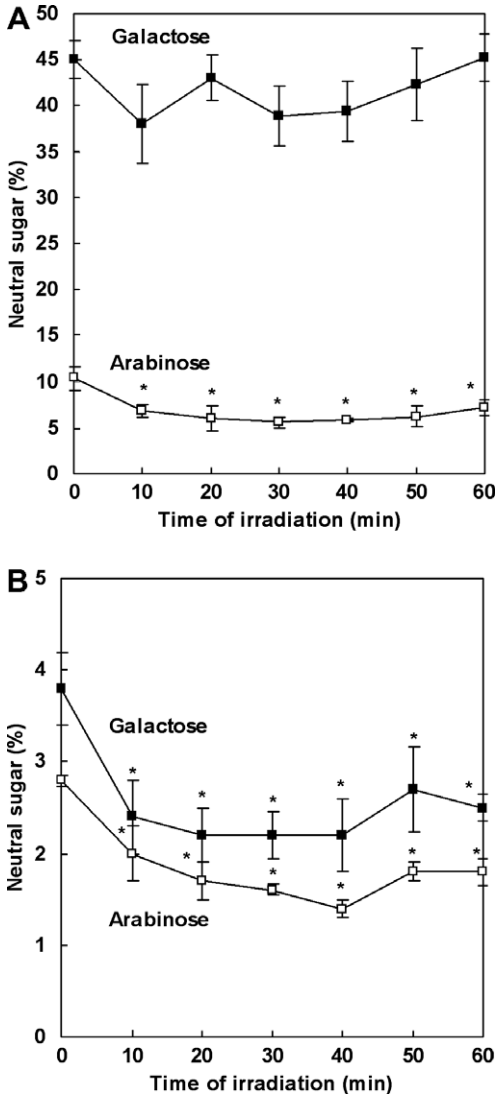

Figure 1. Changes in the sugar compositions of acidic arabinogalactan AG (A) and silenan SV (B) isolated from *S. vulgaris* callus tissue irradiated with UV-C during the exponential phase (12th day) and analyzed during the stationary phase (21st day). The data are expressed as the means from three experiments. Bars indicate s.d., $^{*}p < 0.05$.

unirradiated cells. The molecular weight distribution of SV after irradiation with UV-C was closely related to the control (unirradiated cells). Molecular weight distributions of AG were found to be similar in irradiated and unirradiated cells. Fraction AG-I with an M_w greater than 300 kDa was found to be dominant (yield was 54%). Fractions AG-II with an M_w of 100–300 kDa (yield was 14%), and AG-III with an M_w of 50–100 kDa (yield was 7%) were shown to be minor.

The carbohydrase activities in the callus tissue irradiated during the exponential phase (12th day) were detected during the stationary phase (21st day). The enzymatic activities were markedly altered depending on the duration of exposure to UV-C. Maximal activities of α-L-arabinofuranosidase, pectinesterase, and β-xylosidase were observed after exposure for 10 min, while activities of these enzymes decreased after exposure for 20–60 min (Fig. 2). The activities of polygalacturonase, β-galactosidase, and cellulase were shown to be enhanced by UV treatment, up to maximal activity after exposure for 40 min. The activities of β-galactosidase and α-L-arabinofuranosidase were found to increase reliably in comparison with the control after exposure for 40–60 and 10–40 min, respectively. Pectinesterase and xylanase activities were shown to arise in the callus after UV-C irradiation. The xylanase activity was found to arise after exposure for 40 min, then decline. The appearance of the pectinesterase activity in cells was found to cause a decreased degree of SV methylesterification.

Thus, UV-C irradiation influences the cell-wall-degrading enzymes. A similar conclusion has been made by Barka et al.,[10] who have shown that UV-C treatment induced a reduction in the activity of cell-wall-degrading enzymes such as the carbohydrases (polygalacturonase, pectinesterase, cellulase, xylanase, and β-galactosidase), thereby delaying the cell-wall degradation in tomato fruit ripening. The activities of carbohydrases were shown to increase in the campion callus after some duration of exposure to UV-C in our experiments. These differences appeared to be connected with the nature of the experimental material and the low initial level of the cell-wall-degrading enzymatic activities in the callus cells.

The reduction in the amounts of galactose and arabinose residues in silenan and of arabinose in arabinogalactan observed during the stationary phase can imply changes in the side chains of polysaccharides. These data suggested that the arabinose residues might release from the side chains of arabinogalactan and silenan. The decrease in the arabinose residue amounts in AG and SV and the amount of galactose in SV seemed to be connected with an increase in the activity of α-L-arabinofuranosidase and β-galactosidase after treatment with UV-C.

2.3. Effect of UV-C on the compositions of cell-wall polysaccharides and carbohydrase activities after a long period of culturing

Irradiated callus tissue was analyzed in the seventh growth cycle after irradiation. Alterations in the sugar compositions of the polysaccharides, such as the reduction in the amounts of galactose and arabinose residues in silenan and of arabinose in arabinogalactan, were shown to remain over a long period of time in culture. The changes in the chemical characteristics of the polysaccharides are given in Figure 3. The degree of SV methylesterification was found to decrease to 3.3–5.5% in comparison with the control data after a long period of culturing the irradiated cells. The molecular weight distributions of SV and AG were shown to be different

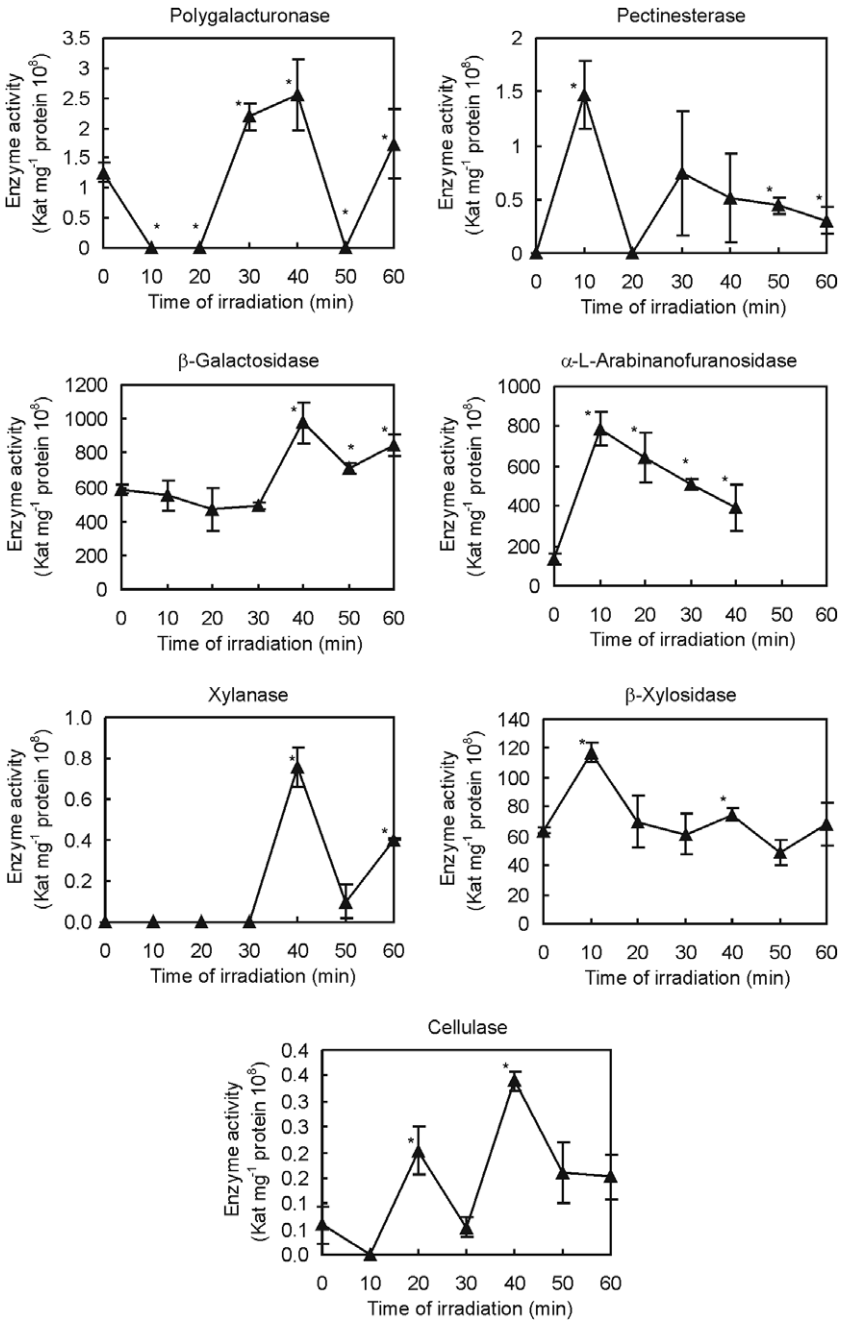

Figure 2. Effect of UV-C on the carbohydrase activities in *S. vulgaris* callus tissue irradiated during the exponential phase (12th day) and analyzed during the stationary phase (21st day). The data are expressed as the means from three experiments. Bars indicate s.d., $^{*}p < 0.05$.

in irradiated and unirradiated cells. The yield of the dominant fraction SV-I, with an M_w greater than 300 kDa, was found to decrease 37% (yield was 47%) in comparison with the control. The yield of the dominant fraction AG-I, with an M_w greater than 300 kDa, was found to rise 30–40% (yield was 70%) in comparison with the control. The yield of the minor fraction AG-II, with an M_w of 100–300 kDa, was shown to decrease 30–40% (yield was 6%). The yield of the minor fraction AG-III, with an M_w of 50–100 kDa (yield was 7%) was closely related to the control.

The reduction in the yield of fraction SV-I in silenan may indicate the destruction of the high-molecular fraction of silenan. The data obtained demonstrate that the molecular weight distributions of AG, such as the increasing yield of the high-molecular fraction AG-I and the decreasing yield of the fraction AG-II with an M_w of 100–300 kDa, present.

The carbohydrase activities in the seventh growth cycle after irradiation were shown to remain high in comparison with the control data (unirradiated cells) (Fig. 4). Namely, β-galactosidase, α-L-arabinofuranosidase,

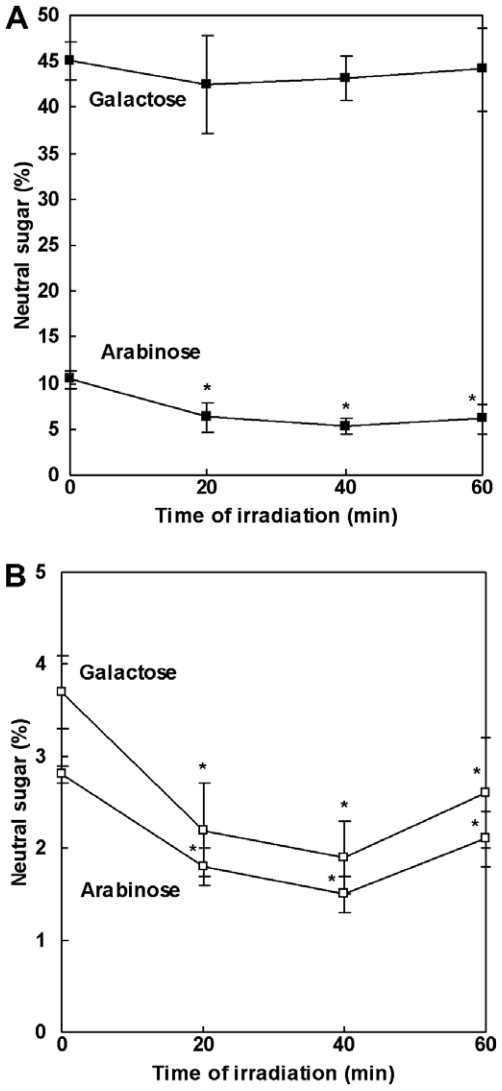

Figure 3. Changes in the sugar compositions of acidic arabinogalactan AG (A) and silenan SV (B) isolated from *S. vulgaris* callus tissue irradiated during the exponential phase (12th day) and analyzed in the seventh growth cycle after irradiation with UV-C. The data are expressed as the means from three experiments. Bars indicate s.d., $^{*}p < 0.05$.

xylanase, and β-xylosidase were found to be maximally active after exposure for 20–40 min; pectinesterase and β-(1→3)-glucanase achieved maximal activity after exposure for 20 min; polygalacturonase and cellulase were the most active after exposure for 40 min.

The effects of UV irradiation range from changes in plant growth and development to more specific effects on primary metabolic functions, such as decreased photosynthetic activity and altered pigment composition and enzyme activities.[13,15] Although there is now a large body of work documenting UV-B effects on various physiological and biochemical processes, little is known concerning the mechanisms of UV effects on these cellular processes. Recent studies have highlighted that UV action leads to profound changes in gene expression and have shown that reactive oxygen species (ROS) play a pivotal role in a number of UV-B signal pathways as the second messenger; increases in these radicals precede increases in other compounds.[15] Increasing ROS levels appeared to be accompanied by the synthesis of salicylic acid (SA), jasmonic acid (JA), and ethylene. Ethylene and SA are involved in the pathway leading to the induction of pathogenesis-related (PR) proteins. Among these PR proteins, carbohydrases such as β-(1→3)-glucanases and chitinases are present.[16] The activities of β-(1→3)-glucanase and other carbohydrases (β-galactosidase, α-L-arabinofuranosidase, α-(1→4)-D-polygalacturonase, pectinesterase, cellulase, xylanase, and β-xylosidase) were shown to be enhanced in the campion callus after treatment with UV-C. Furthermore, the sugar compositions of cell-wall polysaccharides were changed under the influence of UV-C. Carbohydrases have earlier been shown to release oligosaccharides from the cell-wall polysaccharides as signaling molecules of plant cells.[17] A pectic oligosaccharide isolated from soybean cell-wall hydrolysates has been found to be an inductor of phytoalexins in soybean.[17] Carbohydrases appear to be involved in plant resistance against UV-C irradiation.

3. Conclusions

Thus, an incubation period from exponential phase to stationary phase after irradiation was shown to be enough for the occurrence of alterations in the biochemical characteristics of the cell-wall polysaccharides. Namely, reductions in the amounts of galactose and arabinose residues in silenan and of arabinose in arabinogalactan were observed during the stationary phase. A decrease in the degree of SV methylesterification was detected for irradiated callus tissue. These alterations were found to persist over a long period in culture and appeared to be connected with irreversible changes in the cell-wall polysaccharide structures, which seemed to be genetically regulated. The molecular weight distributions of SV and AG were found to be similar in irradiated and unirradiated cells in the stationary phase, while these distributions changed over a long period of time in culture. UV-C irradiation was found to enhance the secretion of some cell-wall-degrading enzymes by the callus, thereby contributing to an alteration in the polysaccharide structure. The appearance of the pectinesterase activity in cells was found to decrease the degree of SV methylesterification.

UV-C irradiation may be employed as a tool for modifying the structural features of the cell-wall polysaccharides. This work is considered to be useful for further regulation of the biosynthesis and production of polysaccharides by cell cultures and for elucidation of plant responses to UV-C stress.

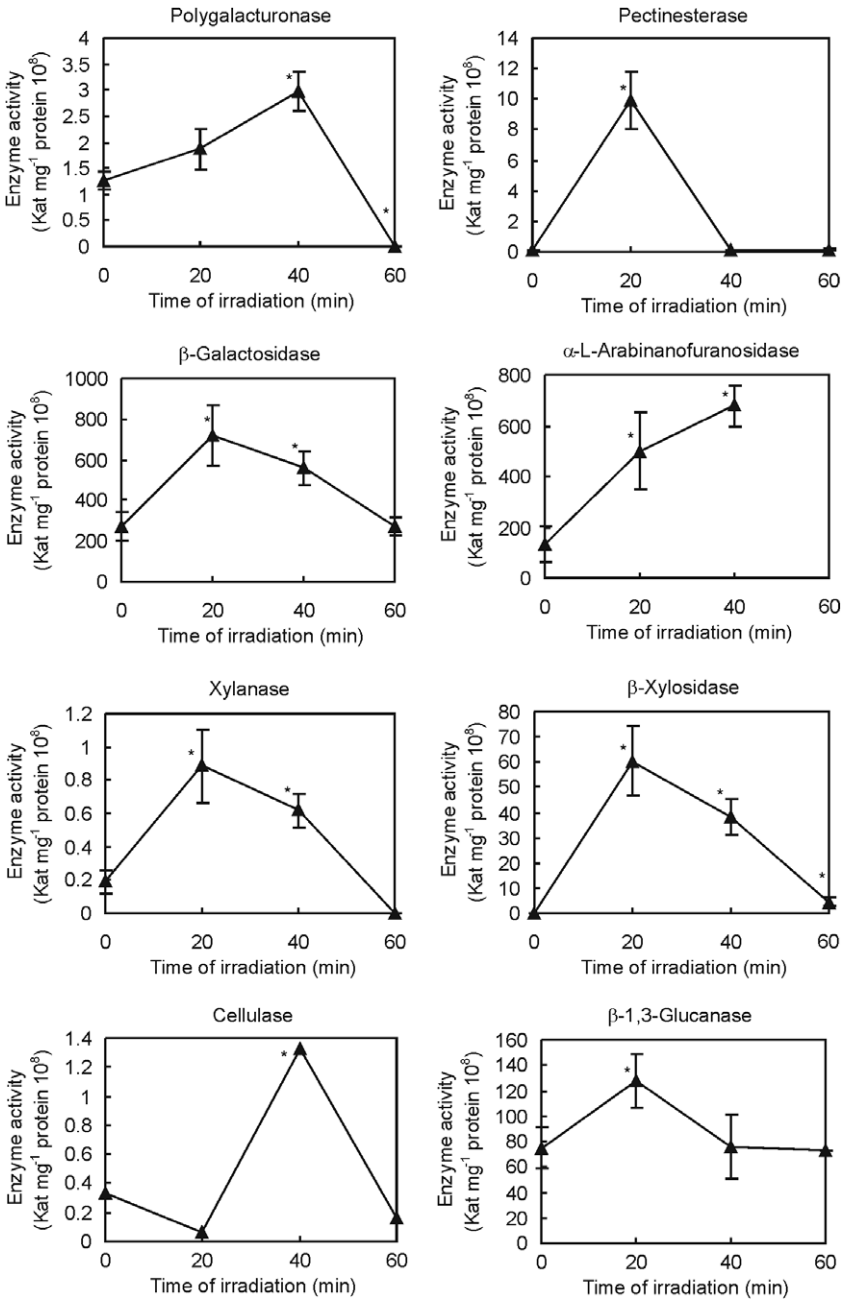

Figure 4. The carbohydrase activities in callus tissue in the seventh growth cycle after irradiation with UV-C. The data are expressed as the means from three experiments. Bars indicate s.d., $^{*}p < 0.05$.

4. Experimental

4.1. General methods

Total amounts of glycuronic acids in polysaccharide fractions were estimated using a reaction with 3,5-dimethylphenol in the presence of concentrated sulfuric acid.[18] Total protein content was determined according to the Lowry method.[19] The degree of methylesterification was calculated using the method described earlier.[20] Spectrophotometric measurements were made with an Ultrospec 3000 instrument (UK). GC was performed with a Hewlett–Packard 4890A chromatograph (USA) fitted with an RTX-1 (0.25 mm × 30 m, 'Restek') capillary column with argon as a carrier gas, using a flame-ionization detector and HP 3395A integrator.

The isolation of polysaccharides was performed as described earlier.[8] The yields were calculated in relation to the dry weight of the callus. Volumetric production per liter of medium of acidic arabinogalactan (AG) and silenan (SV) was estimated. The data obtained are expressed as the means from three separate experiments.

4.2. Plant material and exposure to UV-C radiation

The callus cultures of *S. vulgaris* were maintained on modified Murashige and Skoog's[21] solid medium. The callus cells were subcultured for 21 days at 26 °C in the dark. The calluses were irradiated for 10, 20, 30, 40, 50, or 60 min with UV-C radiation (254 nm, 7 mW m^{-2}). Exposure to UV-C radiation was carried out during the exponential growth phase (on day 12) and analyzed at the stationary phase (on day 21) and in the seventh growth cycle after irradiation. Unirradiated cells were maintained in the dark and used as a control.

4.3. Complete acidic hydrolysis

Polysaccharide samples of AG and SV (2 mg) were hydrolyzed with 2 M TFA (0.5 mL) at 100 °C for 3–4 h in sealed tubes as described earlier.[3,4] The acid was removed by repeated co-evaporation with MeOH. The neutral sugars were quantified by GC as the corresponding alditol acetates using *myo*-inositol as the internal standard.[22] The molar ratios were calculated from the peak areas.

4.4. Molecular weight distributions of SV and AG

Each fraction of SV and AG (30 mg) was dissolved in distilled water (50 mL) and separated successively using various ultrafiltration membranes as follows: polysulfone 300, 100, and 50 kDa (Millipore, USA). The fractions were concentrated and lyophilized to furnish the purified SV-I and AG-I (M_w more than 300 kDa), SV-II and AG-II (M_w 100–300 kDa), and SV-III and AG-III (M_w 50–100 kDa).

4.5. Assay of carbohydrases

Fresh biomass (10 g) was homogenized in 20 mL of sodium acetate buffer (pH 5.0, 0.05 M) for 5 min using a mortar and pestle, followed by extraction for 2 h at 4 °C. Homogenate was centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatant was used as the crude extract for the assay of carbohydrases.

The carbohydrase activities in the callus irradiated during the exponential growth phase (12th day) were detected during the stationary phase (21st day) and in the seventh growth cycle after irradiation. The activities of polygalacturonase (total), pectinesterase (3.1.1.11), β-galactosidase (3.2.1.23), β-(1→3)-glucanase (3.2.1.39), α-L-arabinofuranosidase (3.2.1.55), cellulase (3.2.1.4), β-xylosidase (3.2.1.37), and xylanase (3.2.1.32) were determined. The data are expressed as the means from three experiments. The enzyme activity is expressed in terms of kat (mol s^{-1}) per mg protein (kat mg^{-1} protein).

Polygalacturonase and xylanase activities were measured by the release of reducing sugars from polygalacturonic acid (ICN) and xylan (Sigma) per s, respectively. Reducing sugars were assayed according to the procedure of Nelson.[23] One unit of polygalacturonase activity was defined as the amount of enzyme that liberated 1 mol of reducing sugar per s at pH 4.6 and 50 °C.[24] One unit of xylanase activity was defined as the amount of enzyme that produced 1 mol of reducing sugar per s at pH 5.0 and 50 °C.[25]

The β-galactosidase and α-L-arabinofuranosidase activities were determined spectrophotometrically at 400 nm using 2-nitrophenyl β-D-galactopyranoside (Sigma) and 4-nitrophenyl α-L-arabinofuranoside (Sigma) as the substrates, respectively. One unit of β-galactosidase or α-L-arabinofuranosidase activity was defined as the amount of enzyme that cleaves 1 mol of substrate per s at pH 4.2 and 30 °C.[26]

β-(1→3)-Glucanase activity was measured by the release of reducing sugars from the β-(1→3)-glucan, namely, laminaran (Sigma). Reducing sugars were assayed according to the procedure of Nelson.[23] Laminaran (0.1%) in 0.05 M sodium acetate buffer (pH 5.0) was used as a substrate. The reaction mixture containing the crude enzyme extract and laminaran was incubated for 40 min at 37 °C.[27] Glucose was used as a standard. One unit of β-(1→3)-glucanase activity was defined as the amount of enzyme that produced 1 mol of reducing sugar per s under the above conditions.

The β-xylosidase activity was analyzed spectrophotometrically at 400 nm using 4-nitrophenyl β-D-xylopyranoside (Sigma) as a substrate. One unit of β-xylosidase activity was defined as the amount of enzyme that cleaves 1 mol of substrate per s at pH 4.0 and 50 °C.[28]

The cellulase activity was assayed according to the procedure reported by Rodionova et al.,[29] using cellulose as a substrate. Reducing sugars were assayed according to the procedure of Nelson.[23] One unit of cellulase activity was defined as the amount of enzyme that produced 1 mol of glucose per s at pH 5.0 and 50 °C.[29]

The pectinesterase activity was analyzed by the titrimetric determination of the carboxyl groups formed upon saponification of the methyl ester of galacturonan. The reaction mixture containing the crude enzyme extract and apple pectin (1%) was incubated for 60 min at 30 °C, then titrated by NaOH (0.1 N) up to pH 7.5.[30] One unit of pectinesterase activity was defined as the amount of enzyme that catalyzed saponification of 1 equiv of the ester bonds in pectin per s at 30 °C.

4.6. Statistical analysis

The results are given as mean ± s.d. The significance of differences between means was evaluated by the Student's *t*-test.

Acknowledgments

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