Effects of homobrassinolide in barley callus culture

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ABSTRACT

Thirty-day-old barley (*Hordeum vulgare* L. cv. Zafer-160) calli were treated with 0, 0.5, 1 μmol/L 22(S),23(S)-Homobrassinolide (HBL) for four weeks. Transfer of calli to hormone-free or HBL-supplemented medium caused an increase in total soluble protein and DNA contents and a decrease in superoxide dismutase (SOD; E.C. 1.15.1.1) activity. 0.5 μmol/L HBL caused the highest protein and DNA levels and the minimum decrease in fresh weight; while the lowest superoxide dismutase activity was recorded in 1 μmol/L HBL-treated group. Calli developed nodular structures and green cell clusters after transfer onto hormone-free or HBL-supplemented medium. Methylation-sensitive restriction fingerprinting (MSRF) analysis detected a few changes in methylation between control and 0.5 μmol/L groups. Combination of data led us to the conclusion that HBL favoured regeneration and shoot development and may ameliorate tissue culture stress in a mechanism, which is independent of cytosine methylation.

Keywords: brassinosteroids; superoxide dismutase; regeneration; MSRF; methylation

Brassinosteroids (BRs) promote cell elongation, cell division, differentiation and senescence (Müssig 2005). They also increase resistance in plants to a wide spectrum of stresses (Ali et al. 2008, Hayat et al. 2010). Promotion of growth and amelioration of stress by BRs are thought to be associated with modification of antioxidant system and enhanced levels of protein, DNA, RNA and carbohydrate (Vardhini and Rao 1998, Bajguz 2000). Alteration in DNA methylation patterns occur during long-term callus culture. 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, was thought to be responsible in the progressive methylation (Lambé et al. 1997). Global DNA methylation rates were found to be significantly lowered in embryogenic calli rather than non-embryogenic (Chakrabarty et al. 2003). Superoxide dismutase (SOD) activity increased during somatic embryogenesis but decreased during shoot organogenesis (Gupta and Datta 2003). The objective of this study was to investigate effects of homobrassinolide (HBL) on *in vitro* growth and regeneration in barley. For this purpose, fresh weight, total soluble protein, DNA contents, SOD activity and DNA methylation levels were compared between HBL-treated and non-treated calli. Up to our knowledge, this is the first report studying effects of HBL on DNA methylation, callus growth and regeneration in barley.

MATERIAL AND METHODS

Callus induction. Callus was initiated in Murashige and Skoog (MS) medium supplemented with 4 mg/L Dicamba (Sigma, St. Louis, USA) from barley (*Hordeum vulgare* L. cv. Zafer-160) mature embryos. Seeds were surface-sterilized in 20% commercial bleach for 30 min. Mature embryos were removed and cultured for 30 days in a programmed growth chamber (25°C, 16/8h light/dark).

HBL treatment. Thirty-day-old calli were cultured on MS medium supplemented with 0, 0.5 (0.25 mg/L) as well as 1 μmol/L (0.5 mg/L) HBL (H1267, Sigma, St. Louis, USA) for 4 weeks and transferred onto hormone-free medium for regeneration. In the control group, 10 μL ethanol (solvent) was used instead of HBL. Fresh weights of calli were recorded on the 0^{th}, 7^{th} and 14^{th} day of the treatment.

Determination of total soluble protein and DNA levels. Calli were crushed in 5 volumes of ice-cold phosphate buffer (0.1 mol/L, pH 7.0) using a pre-chilled porcelain mortar and pestle. An aliquot of homogenate was used to determine DNA content. Remaining homogenate was transferred into cold centrifuge tubes and centrifuged at 15 000 × g, 4°C for 20 min. Supernatants were used to estimate total soluble protein and SOD levels.

Supported by the Fund of Istanbul University, Projects No. 3618 and UDP-18399.

Total soluble protein contents were determined according to Lowry et al. (1951) using bovine serum albumin (G5009, Sigma, St. Louis, USA) as protein standard.

DNA contents were determined according to Burton (1956) with modifications (Nigam and Ayyagari 2007) using herring DNA (K1089, Fluka AG, St. Louis, USA) as standard. Briefly, 100 µL non-centrifuged homogenate were mixed with 100 µL perchloric acid (5%), incubated at 70°C for 15 min and then 400 µL diphenylamine reagent (freshly prepared) were added and incubated in a boiling waterbath for 15 min. After cooling to room temperature, absorbance values of the samples at 595 nm were recorded. To prepare diphenylamine reagent, 250 mg of diphenylamine (D2385, Sigma, St. Louis, USA) was dissolved in 25 mL acetic acid and 687 µL sulfuric acid was added just before use. Total soluble protein and DNA levels were expressed as protein or DNA content per g fresh tissue (mg/g).

Determination of SOD activity. SOD activity was measured spectrophotometrically based on inhibition of photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). One mL reaction mixture containing 50 mmol/L phosphate buffer (pH 7.0), 50 mmol/L sodium carbonate, 0.1 mmol/L EDTA, 13 mmol L-methionine, 75 µmol/L NBT (Sigma, St. Louis, USA) and 2 µmol/L riboflavin and 2 µL of 5 × diluted extract. Samples were illuminated with three 15-W white fluorescent lamps for 30 min at room temperature. An enzyme-free sample was used as positive control. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction at 560 nm. Enzyme activity was expressed as enzyme unit per µg protein per min (U/µg protein/min).

Methylation-sensitive restriction fingerprinting (MSRF). Three calli for each group were selected. Genomic DNAs were isolated according to Kidwell and Osborn (1992) and Khanuja et al. (1999) from control and 0.5 µmol/L groups and bulked. Bulked DNA samples were divided into 2 groups. One group was restricted with *Mse*I (ER0981, Fermentas, Maryland, USA) only; the other group was restricted with *Mse*I and then *Bst*UI (ER0921, Fermentas), according to the manufacturer's recommendations. Digested samples were purified using kit (11732668001, Roche, Indianapolis, USA). Purified digestion samples were amplified with 3 primer combinations using 4 primers (Table 1). Appr. 100 ng digested DNA was amplified in a 20 µL reaction mix consisting of 1 × buffer (60 mmol/L Tris-SO_4, 20 mmol/L $(NH_4)_2SO_4$, 3% glycerol, 0.06% NP-40, 0.05% Tween-20, 2 mmol/L $MgSO_4$, pH 9.0), 5% DMSO, 0.05 mmol/L each dNTP, 0.4 µmol/L (8 pmol) each primer (Davies 2002), 0.5 U Taq polymerase (FIREPol® DNA Polymerase, Solis BioDyne, Tartu, Estonia). Amplification conditions were as follows; initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 38°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min.

Polyacrylamide gel electrophoresis. Ten µL aliquot of amplification products were mixed with 2 µL loading dye (100 mmol/L EDTA, pH 7.6, 1% SDS, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol) and incubated at 65°C for 10 min and transferred onto ice. Amplification products were resolved on a non-denaturing, 8% polyacrylamide (19:1 acrylamide:bisacrylamide) gel at 200 V for 5 h in 1 × TBE buffer (89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L EDTA, pH 8.0) and detected by silver staining. Band sizes were determined by comparison with a 50 bp DNA ladder (Favorgen, Ping-Tung, Taiwan). Distinct bands were scored and banding patterns were evaluated according to Table 1.

Statistical analyses. All tissue culture experiments were repeated three times with different samples. Two-tailed and type 3 'TTEST' (Student's *t*-test) were used to estimate the statistical significance ($P = 0.05$) of data.

RESULTS

Morphology. HBL-treated calli, especially 0.5 µmol/L HBL-treated, were bigger, more compact and more friable than control and showed slightly more yellowish morphology (Figure 1). Green cell clusters formed in all groups after 14–15 days. All groups developed shoots but the longest shoots were observed in 0.5 µmol/L group. Some calli in control group also developed roots. However, rooting of the regenerated shoots could not be achieved. Even control group calli could not sur-

Table 1. Possible outcomes of methylation-sensitive restriction fingerprinting (MSRF) technique (Huang et al. 1997)

Condition	Methylation	Control		0.5 µmol/L	
		*Mse*I	M*se*I/ *Bst*UI	*Mse*I	M*se*I/ *Bst*UI
1	no methylation	+	–	+	–
2	normal methylation (or no *Bst*UI sites)	+	+	+	+
3	hypermethylation	+	-	+	+
4	hypomethylation	+	+	+	–

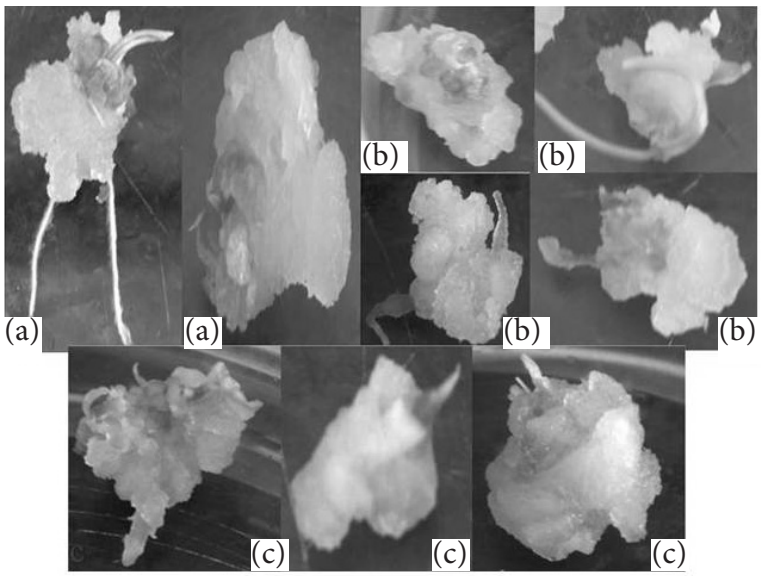

Figure 1. Regeneration in barley calli. (a) control; (b) 0.5 µmol/L homobrassinolide (HBL)-treated; (c) 1 µmol/L HBL-treated calli

vive on hormone-free media after subculturing. Shoots etiolated and calli became brownish after transfer onto hormone-free media.

Fresh weight. HBL-supplemented media decreased fresh weight as well as hormone-free medium (Table 2). Hormone-free medium and 1 µmol/L HBL both caused dramatic decrease in fresh weight during the first 7 days. Fresh weight did not change in these 2 groups after 7 days. There was a strong positive correlation between control and 1 µmol/L HBL-treated calli ($r(1) = 0.910$, $P > 0.05$).

Protein, DNA contents and SOD activity. Thirty-day-old, control and HBL-treated calli were used in estimation of total soluble protein, DNA levels and SOD activity. Total soluble protein and also DNA levels increased after transfer to hormone-free medium as well as HBL-supplemented medium and reached maximum at 0.5 µmol/L HBL concentration (Table 3). Protein ($r(1) = 0.997$, $P = 0.05$) and DNA ($r(1) = 0.998$, $P < 0.05$) contents of control and 1 µmol/L HBL-treated calli were positively correlated. Maximum SOD activity was recorded in 30-day-old calli. Transfer to hormone-free or HBL-supplemented medium decreased SOD activity. SOD activity of control and 1 µmol/L HBL-treated calli was also positively correlated ($r(1) = 0.876$, $P > 0.05$).

Methylation. A1 × A2 primer combination resulted in smear. A1 × A3 and A1 × A4 (Figure 2) combinations amplified 14 and 18 bands in 4 groups, respectively. There were many bands much larger than 500 bp in both combinations. Both combinations especially A1 × A4 amplified many monomorphic bands. A total of ten bands from two primer combinations were analyzed (Table 4). Three of them correspond to regions of which methylation status did not change; two represent hypermethylated regions and one represent hypomethylated region. Four of the bands could not be interpreted and marked with an asterisk. These bands may be PCR artefacts or mutations.

Table 2. Effect of homobrassinolide on fresh weight of calli (mg)

	0^{th} day	7^{th} day	14^{th} day
Control	370 ± 80.2	326 ± 73.5	326 ± 78.3
0.5 µmol/L	240 ± 90.1	223 ± 84.1	203 ± 78.8
1 µmol/L	316 ± 103.9	276 ± 92	266 ± 92.6

DISCUSSION

In this study, regeneration took place both in hormone-free and HBL-supplemented medium. Hormone-free and HBL-supplemented media decreased fresh weight, SOD content; increased protein and DNA levels. Control and HBL-treated calli exhibited quite similar methylation patterns. Brassinolide (BL) and 24-Epibrassinolide (EBL) induced shoot growth while inhibited root growth and increased length, fresh and dry weight in shoots

Table 3. Effects of homobrassinolide on total soluble protein, DNA content (mg/g) and superoxide dismutase (SOD) activity (U/µg protein/min)

	Protein (mg/g)	DNA (mg/g)	SOD (U/µg protein/min)
30 days	1.67 ± 0.07	1.34 ± 0.2	0.179 ± 0.048
Control	2.32 ± 0.43	1.76 ± 0.32	0.06 ± 0.027
0.5 µmol/L	3.01 ± 0.55	2.90 ± 0.47	0.03 ± 0.014
1 µmol/L	1.94 ± 0.59	2.26 ± 0.6	0.03 ± 0.006

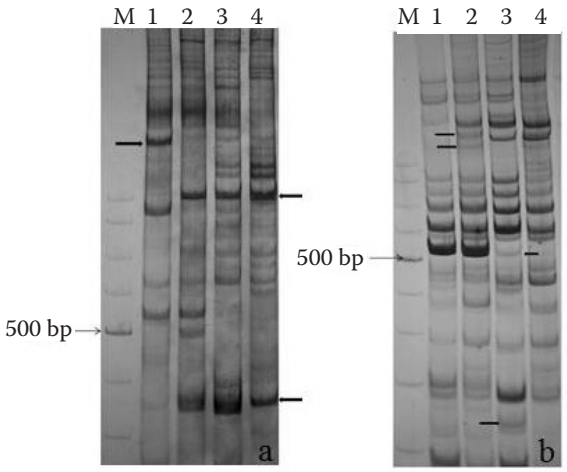

Figure 2. Methylation-sensitive restriction fingerprinting (MSRF) profiles of control and 0.5 µmol homobrassib nolide (HBL)-treated calli with A1 × A3 (a) and A1 × A4 (b) combinations. M – marker; 1 – control/*Mse*I + *Bst*UI; 2 – control/*Mse*I; 3 – (0.5 µmol/L)/*Mse*I; 4 – (0.5 µmol/L)/*Mse*I + *Bst*UI. Arrows indicate some of the polymorphic bands

however decreased in roots (Vardhini and Rao 1998). BRs not only restored but also increased fresh and dry weight in stressed plants (Anuradha and Rao 2007, Arora et al. 2008). BL along with indole-3-acetic acid (IAA) and 6-benzylaminopurine (BA) increased callus fresh and dry weight in *Spartina patens*, a halophyte monocot (Lu et al. 2003). BR treatment was also negatively effective on the fresh weight of calli in cotton (Aydin et al. 2006). BL along with IAA and BA increased shoot regeneration and shoot height in *Spartina patens* (Lu et al. 2003). BR stimulated somatic embryogenesis in cotton (Aydin et al. 2006). BRs increased protein content and CAT and SOD activity in *Brassica juncea* L. under field conditions (Sirhindi et al. 2009). However, HBL decreased protein content and SOD, catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.11) activities in germinating barley embryos (Kartal et al. 2009). BRs were shown to increase protein and nucleic acid content, antioxidant activity and alleviate negative effects of stress (Anuradha and Rao 2007, Arora et al. 2008). Plant tissue culture, mainly callus phase, was defined as a stress factor (McClintock 1984). The activity of one or more of antioxidant enzymes is generally increased in plants exposed to stressful conditions (Ali et al. 2011). Cytokinins, which are used to induce shoot organogenesis, were proposed to act as radical scavengers (Pauls and Thompson 1982). Gupta and Datta (2003) reported the decrease in SOD activity during shoot organogenesis. Auxin-both natural and synthetic-levels in medium had a strong effect on the level of 5-methyl-cytosine in the DNA of cultured

Table 4. Band profiles of methylation-sensitive restrich tion fingerprinting (MSRF) primer combinations

Combination	Band (bp)	C/M	C/M + B	0.5/M	0.5/M + B	Result
	> 500	–	–	+	+	*
	> 500	–	–	+	+	*
A1 × A3	500	+	–	+	+	3
	360	+	+	+	+	2
	225	+	–	+	+	3
	> 500	+	–	–	–	*
	400	+	+	+	+	2
A1 × A4	350	+	+	+	+	2
	315	+	+	+	–	4
	125	–	–	+	–	*

C/M – control/*Mse*I; C/M + B – control/*Mse*I + *Bst*UI; 0.5/M – (0.5 µmol/L)/*Mse*I; 0.5/M + B – (0.5 µmol/L)/*Mse*I + *Bst*UI; + present; – absent

carrot cells (LoSchiavo et al. 1989). It was suggested that oxygen radicals induce alterations in cytosine methylation (Weitzman et al. 1994). Huang et al. (1997) stated that MSRF detects at least four conditions related to DNA methylation. MSRF detected a few changes in methylation and unexpected bands. However, MSRF with these primer combinations provide sufficient number of bands and therefore might be useful to study methylation variations in plant systems. According to our knowledge, there is no report about cytosine methylation in presence of BRs. Callus induction and organogenesis have been studied and optimized in barley (Kachhawa et al. 1997, Yadav et al. 2011). However knowledge on physiological and molecular effects during callus formation and regeneration is limited. *In vitro* effects of HBL in barley has not been studied. In conclusion, we suggest that Dicamba caused stress, free radical generation and callus formation. However, elimination of auxin or replacement with HBL alleviated tissue culture stress, disabled antioxidant system and therefore led to regeneration/shoot organogenesis independent of cytosine methylation. We could achieve only shoot organogenesis. Nevertheless, more detailed research *i.e.* investigation of other antioxidant enzymes, RNA, carbohydrate content, changes in dry weight; testing of different concentrations and rooting of regenerated plants is required to elucidate *in vitro* effects of BRs.

Acknowledgements

We thank two anonymous reviewers for their valuable suggestions.

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Received on May 7, 2012

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