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ORIGINAL ARTICLE

Expression of the gene for ribosome-inactivating protein, *SoRIP2*, as a tool for the evaluation of somatic embryogenesis in spinach

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Abstract High variability in regeneration capacity has previously been observed within a population of seedlings in several spinach cultivars. The cultivar "Matador" exhibits particularly low regeneration potential, and the majority of lines obtained in our previous study displayed a stable embryogenic capacity only for a limited period of time. In order to shorten the time required for embryogenic capacity assessment for individual lines, a model system for the rapid evaluation of embryogenic capacity was developed. This model system was based on the expression of a gene encoding spinach ribosome-inactivating protein (*SoRIP2*), which showed low expression levels in roots grown under non-inductive conditions. Induction of globular somatic embryos (SEs) resulted in a 285-fold increase in *SoRIP2* expression that dropped to the control level beyond cotyledonary-stage SEs. The model system was tested by comparing the expression of *SoRIP2* and the index of embryo-forming capacity (EFC), which integrates the frequency of regeneration and the mean SE number per root explant. Comparisons were always made within the same line, and the expression of *SoRIP2* and the EFC index were determined 4 and 12 weeks after starting induction treatment, respectively. High positive correlations between *SoRIP2* expression and EFC were obtained for the two factors that influenced embryogenic capacity the most: genotype ($r^2 = 0.81$) and photoperiod ($r^2 = 0.92$). The results indicate that the expression of *SoRIP2* can be successfully used for early evaluation of regeneration capacity of individual lines, before SEs can be seen with the aid of a stereomicroscope, even 8 weeks earlier than by the conventional method.

Abbreviations

ANOVA	Analysis of variance
BM	Basal medium
EFC	Embryo-forming capacity
GA_3	Gibberellic acid
IM	Induction medium
NAA	α-Naphthaleneacetic acid
Kin	Kinetin (N^6-furfuryl aminopurine)
LD	Long day condition
PGR	Plant growth regulator
PPFD	Photosynthetic photon flux density
SD	Short day condition
SE	Somatic embryo(s)
SoRIP	Ribosome-inactivating protein

Introduction

An efficient and reliable protocol for in vitro regeneration is a prerequisite for molecular breeding of crops, and somatic embryogenesis has been recognized as a superior process of de novo regeneration. In spinach (*Spinacia oleracea* L.), high variation in somatic embryo-forming capacity (EFC) has been found among randomly chosen individuals, both in cv. "Nippon" (Ishizaki et al. 2001) and "Matador" (Milojević et al. 2011). As a consequence, the genotype at the individual plant level has a strong effect on the

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assessment of other factors affecting somatic embryogenesis (Milojević et al. 2012), and must not be overlooked. In addition, cv. "Matador", which is highly important for the Serbian market, exhibits extremely low embryogenic capacity since individuals with high regeneration capacity are rare in a population (Milojević et al. 2011). Hence, testing and screening for individuals with high embryogenic capacity prior to any research is highly advisable. Given that the induction of somatic embryogenesis is labor-intensive and time-consuming, finding a reliable and robust molecular marker for the early evaluation of embryogenic capacity is of paramount importance.

The expression of early somatic embryogenesis-related genes, like *Wuschel* (*WUS*), *Leafy Cotyledon* (*LEC*), *Baby Boom* (*BBM*), *Somatic Embryogenesis Receptor Kinase* (*SERK*) etc., allows for the determination of the embryogenic potential of explants before any visible morphological changes occur (Zuo et al. 2002; Stone et al. 2001; Boutilier et al. 2002; Schmidt et al. 1997; Mahdavi-Darvari et al. 2015). Unfortunately, to the best of our knowledge, none of these genes have been cloned in spinach to date. However, two spinach proteins, shown to be differentially expressed during somatic embryogenesis, have been cloned and characterized (Ishizaki et al. 2002; Kawade et al. 2008). These two ribosome-inactivating protein genes (*SoRIP1* and *SoRIP2*) have been isolated from embryogenic calli of spinach (Ishizaki et al. 2002), and their expression was found to be differentially regulated in a development-dependent manner during somatic embryogenesis (Kawade et al. 2008). The expression of *SoRIP1* increased in embryogenic calli with the acquisition of embryogenic potential and then gradually decreased during the course of somatic embryo development, whilst the expression of *SoRIP2* was low in embryogenic calli but increased markedly with the development of somatic embryos (Kawade et al. 2008). Immunohistochemistry showed that SoRIP1 preferentially accumulated in the proembryos and peripheral meristem of somatic embryos early in development, while intense accumulation of *SoRIP2* was detected in the epidermis of somatic embryos (Kawade et al. 2008), as well as in immature zygotic embryos (Kawade and Masuda 2009). In the promoter region of *SoRIP1*, an RY motif (a Wuschel-type homeodomain protein binding site) has been found, suggesting its role in the development of the embryo proper (Kawade and Masuda 2009). In the promoter region of *SoRIP2, cis* elements related to plant defense have been found, indicating that SoRIP2 plays a defense-related role (Kawade and Masuda 2009).

Although SoRIP proteins probably do not play a direct role in somatic embryo initiation and development, their expression coincides with somatic embryo formation and could be exploited for an evaluation of embryogenic capacity. To test this hypothesis, SoRIP2 expression was investigated. The aim of the present study was to assess if the expression of *SoRIP2* could be used as a molecular marker for an early evaluation of embryogenic capacity in spinach.

Materials and methods

Basal and induction media

The basal medium (BM) contained full strength macro and micro salts (Lachner, Brno, Czech Republic) according to Murashige and Skoog (1962), 20 g/l sucrose, 100 mg/l myo-inositol, 2 mg/l thiamine, 2 mg/l pyridoxine, 5 mg/l nicotinic acid and 2 mg/l adenine (all purchased from Sigma Aldrich, St. Louis, MO, USA). For gelling, 7 g/l agar (Torlak Institute, Belgrade, Serbia) was added to the medium, and the pH was adjusted to 5.6 before sterilization using a pH meter. The medium was sterilized at 114 °C and 80 kPa for 25 min. For the induction of somatic embryogenesis, 20 µM α-naphthaleneacetic acid (NAA, Sigma-Aldrich) and 5 µM gibberellic acid (GA$_3$, Sigma-Aldrich) were added to the BM, hereafter referred to as induction medium (IM). GA$_3$ was dissolved in absolute ethanol, sterilized by filter sterilization (0.22 µm, Merck Millipore, Billerica, MA, USA) and added to the sterilized medium cooled to approximately 40 °C.

Plant material, regeneration procedure and culture conditions

Spinach seeds cv. "Matador" (NS SEME, Novi Sad, Serbia) were sterilized and germinated as described in Milojević et al. (2011). Briefly, the seeds were washed with tap water and commercial detergent (Fairy, Procter and Gamble, Ohio, USA), and then surface sterilized using 30% bleach solution (4% NaClO, Panonija, Pančevo, Serbia) for 30 min, followed by 15% bleach for 15 min. The seeds were rinsed with sterile distilled water, dried on sterile filter-paper and planted in 90 mm Petri dishes (20 seeds per dish) containing 25 ml plant growth regulator (PGR)-free BM for germination. When the resulting axenic seedlings fully developed 4–5 leaves and a branched root system, they were used as donor material for root explants. Every seedling was considered an individual line, as were all somatic embryos (SEs) that were regenerated from its roots.

For the induction of somatic embryogenesis, 1 cm-long apical fragments of the lateral roots were isolated from seedlings and cultivated on IM for 12 weeks. Explants were subcultured on the same medium at 4-week intervals. After each subculture, SEs regenerated from root fragments were recorded, harvested and transferred to BM supplemented with 5 µM 6-furfurilamino purine (kinetin, Kin,

Sigma-Aldrich) for further development and multiplication. Roots of the SE-derived plants were also used for the induction of somatic embryogenesis, as described for seedlings roots.

Number of SEs was recorded with the aid of a stereomicroscope and embryogenic capacity was estimated using the following parameters: the frequency of explants regenerating SEs, the mean SE number per explant, and the index of SE-forming capacity (EFC), calculated as follows: EFC = (mean SE number per explant) × (% of regenerating explants)/100. These parameters were calculated for the overall 12-week period.

The cultures were maintained under diffuse light provided by cool white fluorescent tubes (Tesla, Belgrade, Serbia), with a photosynthetic photon flux density of $100\ \mu mol\ m^{-2}\ s^{-1}$, as measured by an LI-1400 DataLogger equipped with an LI-190SA Quantum sensor, LI-COR Biosciences, for 16 h per day at $25 \pm 2\,^\circ C$. The explants were exposed to long day (LD, 16 h light and 8 h dark) or short day (SD, 8 h light and 16 h dark) conditions, as specified for each experiment.

RNA isolation and real time PCR

Total RNA was isolated from root tissue, according to the procedure of Gasic et al. (2004). The samples were treated with DNase I (Thermo Scientific, Waltham, MA, USA) at $37\,^\circ C$ for 30 min, according to the manufacturer's instructions.

For each reverse transcription (RT) reaction, 200 ng of total RNA were used per 20 µl reaction mixture, along with 2.5 mM $MgCl_2$, 1 mM dNTP, 10 mM dithiothreitol (DTT), 1.25 mM oligo-dT, 10 U of RNase inhibitor and 15 U of MultiScribe® transcriptase.

Expression levels of *SoRIP2* (GenBank™ accession number AB435547.1; Kawade and Masuda 2009) were measured by quantitative real-time RT-PCR (qRT-PCR) method, in a 25 µl qPCR reaction, containing 2.5 µl of RT reaction product and Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific), and calculated according to the ΔΔCt method (Livak and Schmittgen 2001). Expression of *SoRIP2* was normalized to the expression of *α-Tubulin* (*α-TUB*, GenBank™ accession number M21414.1; Kawade and Masuda 2009). For each sample, qRT-PCR was performed in triplicate. The forward primer 5′-ACC-TTC-CTA-AGT-GAC-ATA-CG-3′ and reverse primer 5′-GCA-TAT-ACT-AAC-GCA-ATT-GG-3′ were used for amplification of *SoRIP2*, while forward primer 5′-ACC-CTC-GGA-TCC-ACT-TCA-TG-3′ and reverse primer 5′-AGG-GCA-GCA-AGA-TCC-TCA-CG-3′ were used for amplification of *α-TUB*. Thermal cycling conditions were: initial denaturation at $95\,^\circ C$ for 5 min, followed by 40 cycles of denaturation at $95\,^\circ C$ for 30 s, annealing at $60\,^\circ C$ for 1 min and extension at $72\,^\circ C$ for 1 min.

Assessment of suitability of *SoRIP2* as a molecular marker

To assess the correct sampling time of the root explants during the day, diurnal variation in expression of *SoRIP2* was tested. In order to minimize individual plant variation, the expression of the *SoRIP2* gene was tested in explants taken from 45 seedlings (three replications, each with 15 seedlings). The apical root fragments (1 cm) were isolated and cultivated on IM, under LD conditions, for 4 weeks, and then collected at different time points during the day: at the moment of turning the light on (time point 0), and after 4, 8, 12, 16 or 24 h i.e. just before turning the light on. RNA samples were prepared from genetically identical plant material for each time point, i.e. an equal number of root explants, taken from each seedling, was used for each time-point sample. Expression levels of *SoRIP2* were measured by qRT-PCR, as described above.

In order to assess the suitability of *SoRIP2* as a marker for the estimation of the embryogenic potential of the explants, its expression was monitored during the course of SE development at distinct stages: globular (≤1 mm), heart-shaped (<2 mm), torpedo-cotyledonary (2–5 mm) and germinating SE (>5 mm). For this analysis, somatic embryogenesis was induced from the apical root fragments of in vitro-grown plants of line 188. Since SEs at the globular stage of development could hardly be seen even with the aid of a stereomicroscope, and could be harmed during preparation, they were sampled together with the root tissue. However, larger SEs were isolated and immediately frozen in liquid nitrogen. Since the highest expression of *SoRIP2* was recorded 4 h after the light was turned on, plant material for molecular analysis was always sampled at that time point. Total RNA was isolated from SEs regenerated from 20 root fragments (five explants in four Petri dishes), which were pooled into a single sample. Analyses were done in three technical replicates. Root fragments cultivated on BM for 4 weeks were used as a control.

Conventional vs. molecular quantification of embryogenic potential

To verify the robustness of *SoRIP2* expression for embryogenic potential quantification, a correlation between its expression and conventionally quantified embryogenic potential was tested in ten randomly chosen lines. For this purpose, the apical root fragments were cultivated on IM for 4 weeks, and then one-half of the root explants was immediately frozen and subsequently used for *SoRIP2* expression quantification, while the other half of the

explants was cultivated for two additional subcultures. The frequency of SE regeneration, the mean SE number per explant and the EFC index were calculated for the overall 12-week period, as described. For both conventional and molecular analyses, four samples (Petri dishes) with five subsamples (root explants) were used for each treatment.

A correlation between the EFC index, obtained after a 12-week period, and the expression levels of *SoRIP2*, analyzed after 4-week period, was tested. Line 203, exhibiting the lowest expression of *SoRIP2*, was used as a control.

As a next step, we investigated the effect of photoperiod on embryogenic potential by conventional and molecular approaches. The root fragments taken from in vitro-grown plants of previously established lines with low (228), average (253) and high (232) embryogenic potential were cultivated on BM or IM, with LD or SD and PPFD of 100 μmol m^{-2} s^{-1} in both photoperiods. Twenty root fragments of each line were used for each treatment (five fragments in each of the four Petri dishes). Conventional and molecular quantification were performed as described above. The fragments of the corresponding line cultivated on BM were used as controls.

Statistical analysis

All percentage data were subjected to angular transformation (arcsin$\sqrt{X}$) and SE number data to square root transformation prior to analysis. After analysis, data were subjected to inverse transformation for presentation. Statistical significance was determined by analysis of variance (ANOVA). The mean values were separated by a Fisher LSD post-hoc test ($p \leq 0.05$). Statistical significance between the gene expression measured in explants of the same line cultivated under LD or SD was estimated using *t* test for dependent samples. The correlation coefficient between EFC and the expression of *SoRIP2* was determined using Pearson correlation coefficient (r^2).

Results and discussion

Before any study of somatic embryogenesis in spinach, the effect of genotype has to be considered. The embryogenic capacity varies greatly among the lines and substantially influences the estimation of the impact of other factors (Milojević et al. 2012). Therefore, a previous characterization of embryogenic capacity of lines to be further used as a donor material is necessary. However, the assessment of regeneration capacity using a conventional approach is labor-intensive and time-consuming and usually took 12 weeks in our experiments. In the majority of lines of cv. "Matador", the differences among the lines with low (Fig. 1a) and high (Fig. 1b) regeneration

capacity were not obvious during the first 4–6 weeks, becoming apparent only after 8–10 weeks (Fig. 1c, d). In addition, SEs are sometimes numerous (Fig. 1e) and embedded in root tissue proliferations (Fig. 1f), making counting SEs difficult, even with the aid of a stereomicroscope. Therefore, a new faster and easier approach for SE number evaluation in spinach is needed.

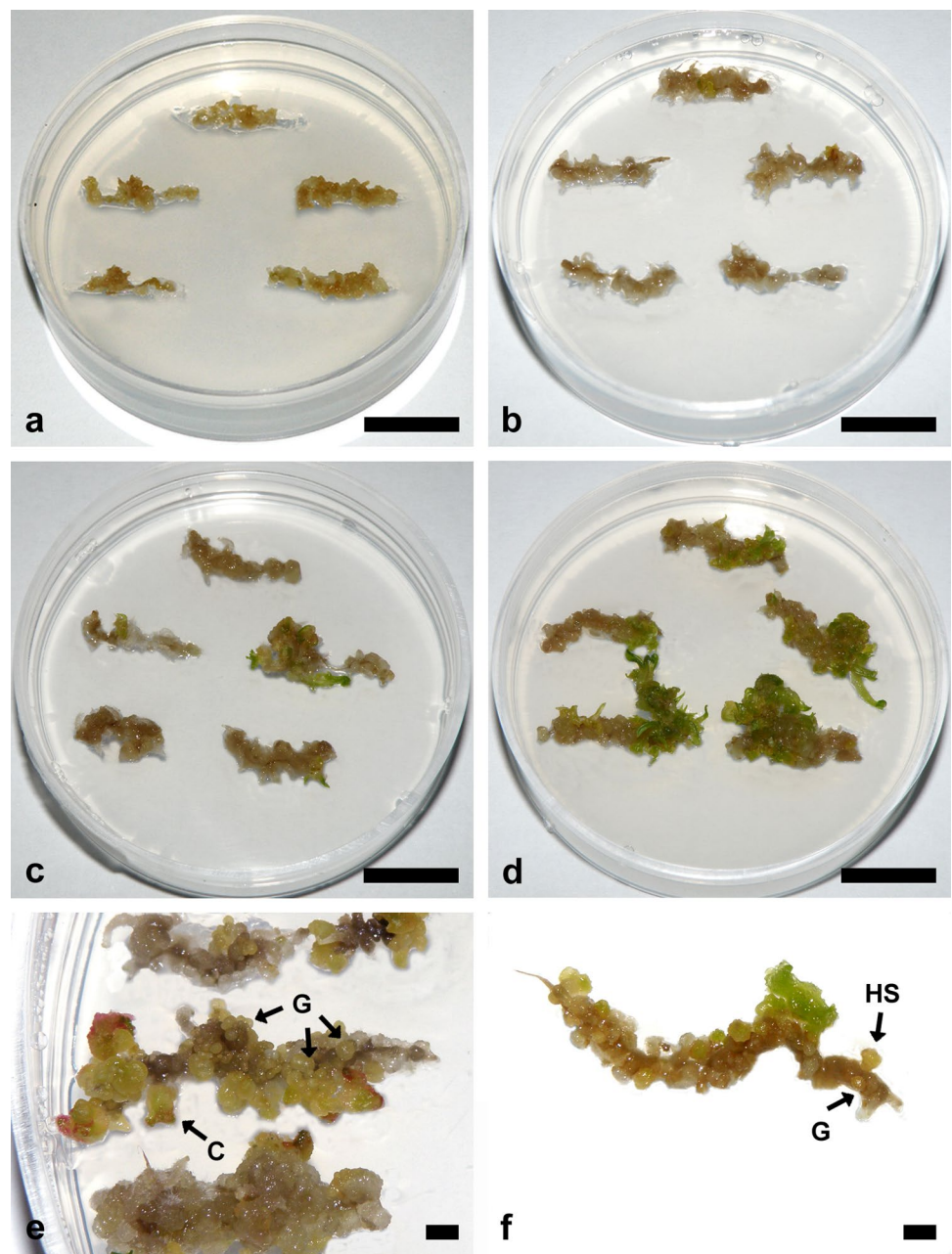### Testing the expression of *SoRIP2* for use as a marker for somatic embryogenesis

The presence of *cis* element within the promoter region of *SoRIP2* gene (Kawade and Masuda 2009), which is known to be responsible for the circadian rhythmicity, required the analysis of its diurnal expression. The highest expression was detected in explants collected 4 h after the beginning of light period, with about a threefold increase compared to the samples collected at time point 0 (Fig. 2). This is in accordance with the general expression pattern of defense-related genes, usually found to be the highest around the middle of the day (Rippmann et al. 1997). As a result, in the present study plant samples were always collected at exactly the same time, 4 h after the lights were switched on.

Stage of SE development significantly affected the expression levels of *SoRIP2* ($p \leq 0.001$), as measured by qRT-PCR. The expression of *SoRIP2* gene was very low in control roots (roots without SEs, cultivated on BM), as was also observed in cv. "Jiromaru" (Kawade and Masuda 2009). However, *SoRIP2* gene expression sharply increased in SEs at the globular stage of development, where it was 285-fold higher than in control. In advanced stages of SE development, which can be visualized with the naked eye (2–5 mm), the expression of *SoRIP2* was twice as low as in globular SEs, while in SEs larger than 5 mm, the expression of *SoRIP2* diminished and returned to the level detected in the control (Fig. 3).

Since the expression of *SoRIP2* peaked in SEs at the globular stage of development, this gene seemed to be a good candidate for the early evaluation of embryogenic capacity in spinach, and this idea was further tested in the continuation of the present study. Our results are consistent with the findings of Kawade et al. (2008) who detected a 440-fold increase in the expression of *SoRIP2* in SEs compared to embryogenic callus, although the stage of SE development was not specified in that study. Similar results were also obtained for SEs of *Larix decidua*, where the highest level of *LdLEC1* gene expression was detected in SEs at the early stage of development, but then decreased in later embryonic stages toward the seedling stage (Rupps et al. 2016).

Fig. 1 Somatic embryo regeneration from the apical root sections of spinach. Roots taken from lines with low (**a**) and moderate (**b**) embryogenic capacity did not differ at the end of the first 4-week subculture, but differences between the lines with low (**c**) and moderate (**d**) embryogenic capacity became apparent after 8 weeks of culture. (**e, f**) Root explants bearing somatic embryos at different stages of development. *G* globular, *HS* heart-shaped, *C* early cotyledonary stage. *Scale bars* **a–d** 1 cm; **e, f** 1 mm


## Testing the robustness of the model system

As the next step, we investigated whether the expression of *SoRIP2* could be used for the determination of the embryogenic capacity of explants. To test this hypothesis, the expression of *SoRIP2* was determined in root explants subjected to induction treatment for 4 weeks and compared to EFC indices calculated for the overall 12-week induction treatment. To avoid the effect of individual variation, the same genetic material (i.e. the same lines) was used for both analyses.

Conventional counting of SEs regenerated from roots of ten randomly selected lines showed that genotype significantly affected SE regeneration frequency, the mean SE number per explant and EFC ($p \leq 0.001$ for all variables) (Fig. 4a–c). All tested lines could be divided into three groups based on the obtained embryogenic capacities: lines with low (203, 204, 228 and 241), average (230, 253 and 268) and high (232, 237 and 240) embryogenic capacity. Line 237 exhibited the highest embryogenic capacity, with an EFC index of 31.06 (Fig. 4c). The frequencies of SE regeneration were high, except in lines with low embryogenic capacity (Fig. 4a), while much wider variation was observed in the mean SE number per explant (Fig. 4b). Therefore, the latter parameter more directly influenced embryogenic capacity (as reflected by the EFC index).
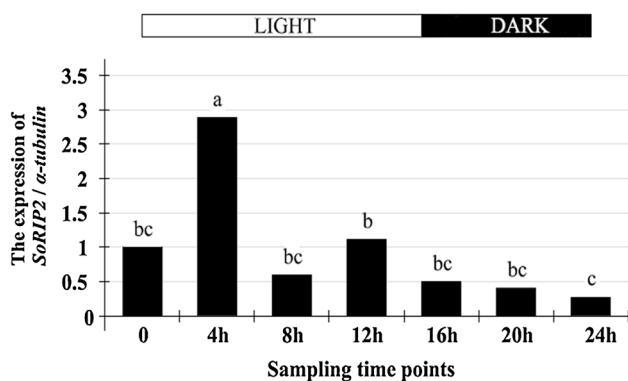
Fig. 2 Diurnal variation in the expression of *SoRIP2* in the apical root fragments cultivated on IM for 4 weeks. Sampling of the material was done at different time points: at the moment of turning the light on (time point 0), and after 4, 8, 12, 16, 20 or 24 h, i.e. just before turning the light on. The explants sampled at the time point 0 were used as a control (=1). *White and black bars* above the chart represent light and dark period, respectively
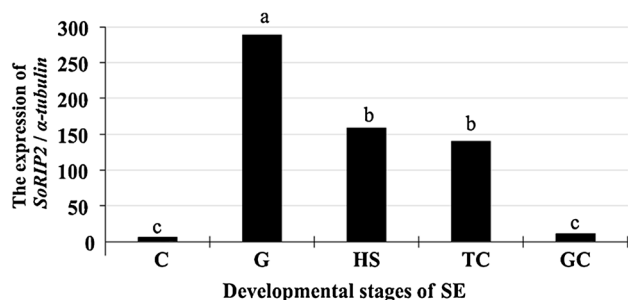
Fig. 3 The expression of *SoRIP2* in SEs at different stages of development. Apical fragments of the lateral roots of line 188 were cultivated on IM and samples were collected when SEs reached different developmental stages. Lateral roots of the same line cultivated on BM were used as a control (=1). The expression was analyzed by the qRT-PCR method and normalized to the expression of α-tubulin. Data represent mean values of three technical replicates. The values in the graph denoted with the *same letters* are not significantly different (p ≤ 0.05) according to the LSD test. *C* control, *G* globular, *HS* heart-shaped, *TC* torpedo-cotyledonary, *GC* germinating stage of development

These results are consistent with our previous findings (Milojević et al. 2011).

Such variability of in vitro regeneration capacity has been observed in many plant species, including soybeans (Song et al. 2010) and *Arabidopsis* (Motte et al. 2014). Song et al. (2010) identified six quantitative trait loci associated with somatic embryogenesis in soybeans, two of which were associated with the frequency of embryogenesis, while the remaining four were associated with SE number per explant. Therefore, the capacity for regeneration is considered to be a quantitative trait. In line with this, small genetic variations in genes associated

with regeneration capacity are proposed to contribute to its great variability (Ikeuchi et al. 2016). However, the capacity for regeneration is associated with genes encoding for an array of different functions in different plant species (Nishimura et al. 2005; Motte et al. 2014; Kareem et al. 2015; de Siqueira Pinto et al. 2017), and it is still unknown how their expression is correlated with meristem initiation. In the case of spinach, in which gibberellins seems to play a pivotal role in the induction of regeneration, the level of endogenous gibberellins in root explants could account for great individual variability of embryogenic capacity, since in some species a reduced level of endogenous bioactive gibberellin enhances somatic embryogenesis (Wang et al. 2004).

Genotype also significantly affected the expression level of *SoRIP2* (p ≤ 0.001). Lines with low embryogenic capacity showed up to 2.5-fold higher expression of *SoRIP2* than the control, with line 203 showing the lowest *SoRIP2* expression (Fig. 4d), while in lines with average embryogenic capacity (230, 253 and 268), an increase in *SoRIP2* expression of about 10-fold was observed. However, in lines with high embryogenic capacity (232, 237 and 240), the expression of *SoRIP2* was more than 20-fold higher than in the control. The greatest increase in the expression level of *SoRIP2* was observed in line 237 (~50-fold, Fig. 4d), which also showed the highest EFC index (Fig. 4c). Consequently, the shapes of the two graphs presenting EFC (Fig. 4c) and the expression of *SoRIP2* (Fig. 4d) were very similar.

Finally, Pearson's correlation coefficient showed a high positive correlation ($r^2 = 0.81$, p ≤ 0.05) between EFC and the expression of *SoRIP2*, confirming that *SoRIP2* could be used as a good molecular marker for estimation of spinach embryogenic capacity. It is important to emphasize once again that the expression of *SoRIP2* was determined after 4 weeks, while EFC was estimated after 12 weeks of cultivation. Therefore, using the expression of *SoRIP2*, spinach embryogenic capacity was adequately estimated 8 weeks earlier than by the conventional method.

In a previous study, an essential effect of light, both in terms of light duration and intensity, on embryogenic capacity in spinach was demonstrated (Milojević et al. 2012). Therefore, in the present study, the impact of photoperiod on the expression of *SoRIP2* was tested and compared to the EFC index. For this experiment, more plant material was needed, so the apical root sections of in vitro-grown plants were used, which have similar embryogenic capacity as roots isolated from seedlings (Milojević et al. 2011). Three lines with various embryogenic capacities (line 228-low, line 253-average and line 232-high, Fig. 5) were chosen for this experiment. The frequencies of SE regeneration and EFC indices were significantly higher (p ≤ 0.05) in LD compared to SD in all tested lines (Fig. 5a, c), while the mean SE number per explant was significantly
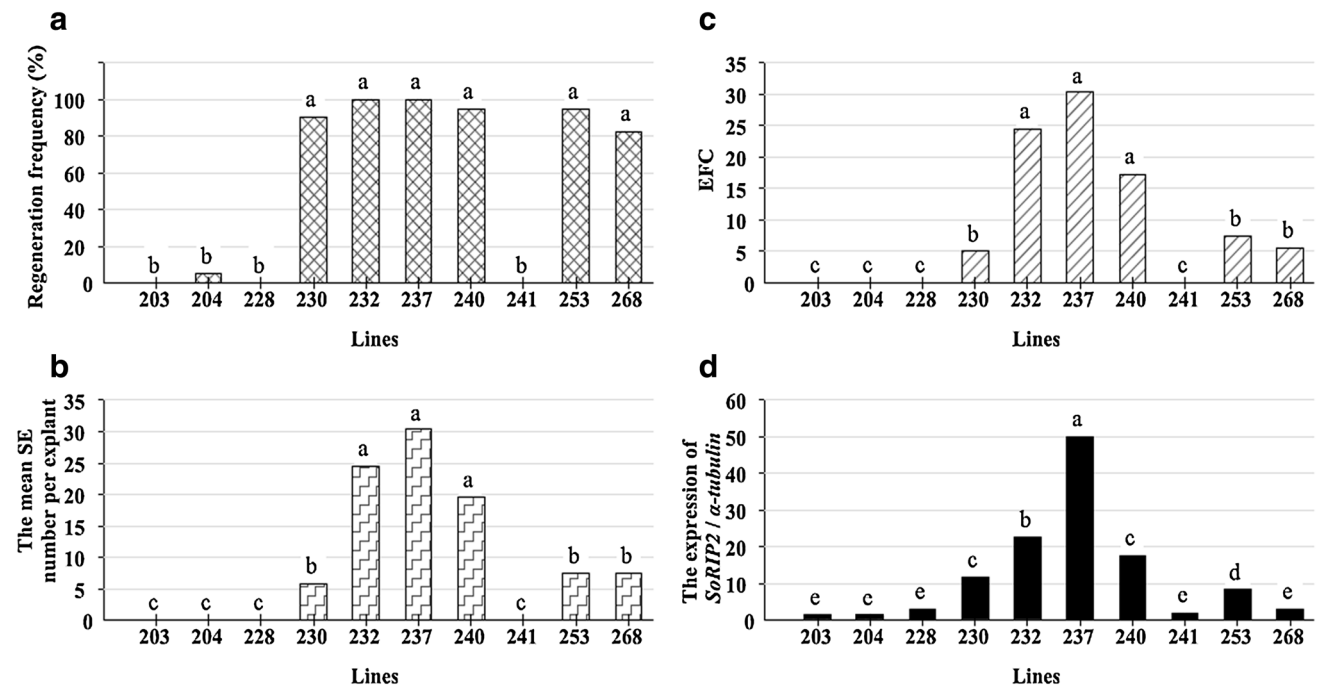
Fig. 4 Effect of genotype on embryogenic capacity in spinach, reported by conventional vs. molecular determination. The frequency of SE regeneration (**a**), the mean SE number per explant (**b**), and the EFC index (**c**) were determined after 12 weeks, while the expression of *SoRIP2* (**d**) was determined after 4 weeks of culture on IM. Data represent mean values. Four Petri dishes with five root fragments (n = 20) were set for each line for both conventional and molecular quantification. The values on the graph denoted with the *same letters* are not significantly different (p ≤ 0.05) according to LSD test

higher in LD in lines 228 and 253, but not in line 232 (p = 0.44, Fig. 5b).

In accordance with this, genotype (p ≤ 0.001), photoperiod (p ≤ 0.05), as well as their interaction (p ≤ 0.001) significantly affected the expression of *SoRIP2*. Furthermore, the expression of *SoRIP2* was significantly higher in all tested lines under LD conditions (Fig. 5d), and Pearson's correlation coefficient showed a strong positive correlation ($r^2 = 0.92$) between EFC and the expression of *SoRIP2*. These results contribute to the robustness of this model system, implying that the expression of *SoRIP2* could be used to test other factors affecting spinach somatic embryogenesis.

Although the most desirable markers for somatic embryogenesis are those specifically expressed in the earliest phase of vegetative-to-embryonic transition, only a few genes meet this criterion *sensu stricto* (Mahdavi-Darvari et al. 2015; Rupps et al. 2016). These genes, such as those encoding transcription factors *WUS, BBM* and *LEC*, are directly involved in the transition of somatic cell to embryogenic cell. However, many candidate genes have been shown to have an array of other functions (Hu et al. 2005; Nolan et al. 2009), and have been considered markers of cell pluripotency and proliferation (Savona et al. 2012). For many proteins differentially expressed in embryogenic cultures, the ability to serve as markers for the acquisition of embryogenic competence or specific embryogenic developmental stage has been shown (Tchorbadjieva et al. 2005; Xu et al. 2011; Pilarska et al. 2013; Tchorbadjieva 2016). In the present study, *SoRIP2* also proved to be a very useful marker for the assessment of embryogenic capacity of spinach cv. "Matador" root sections.

The importance of the presented approach is further emphasized by the fact that only a few spinach lines regenerated SEs during a 4-week period of cultivation on IM, while in most of the lines the first signs of SE regeneration became apparent between the 6th and 8th week of cultivation (Milojević et al. 2011). The first observed SEs could be visualized only by a stereomicroscope, so screening for the lines with high embryogenic capacity was very labor intensive. Additionally, embryogenic capacity decreases with time in most lines, hence it is highly advisable to reduce the time needed for the line characterization (Milojević et al. 2011). Thus, determining the expression of *SoRIP2* could be a reliable and much faster method for screening embryogenic capacity, providing the possibility to speed up research and to use only the lines whose regeneration capacity is still high. Furthermore, identifying the lines with high embryogenic
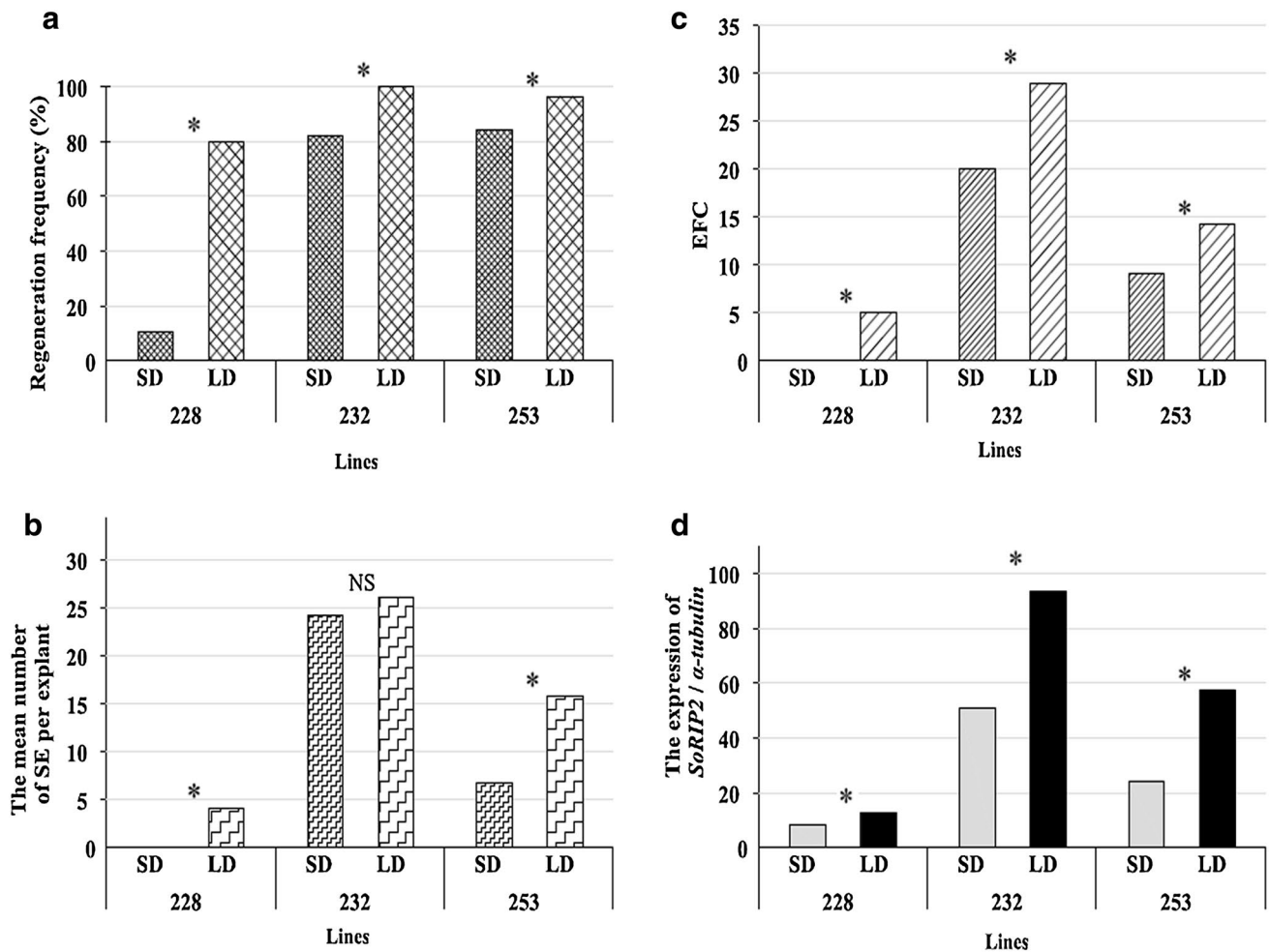
Fig. 5 Effect of photoperiod on embryogenic potential in spinach, reported by conventional vs. molecular determination. Apical root sections were isolated from three in vitro-grown plants of lines 228, 232 and 253. Roots were cultured on IM under LD or SD conditions. The frequency of SE regeneration (**a**), the mean SE number per explant (**b**), and the EFC index (**c**) were recorded after 12 weeks, while the expression of *SoRIP2* (**d**) was determined after 4 weeks of culture. Data represent mean values. Four Petri dishes with 5 root fragments (n=20) were set for each line and for each photoperiod. LD and SD treatments for the same line were compared by *t* test for dependent samples. *LD* long day conditions, *SD* short day conditions, *NS* non-significant, *Significantly different at p≤0.05

capacity enables us to obtain lines with extremely high embryogenic capacity by in vitro self-fertilization (Milojević et al. 2011).

Author contributions JM and SZ-K designed the study; JM, LjT, ND and DĆ performed tissue culture experiments; MM, JM and JS performed molecular analyses; JM and MM performed statistical analyses and prepared figures; JM wrote the manuscript and SZ-K and JS contributed in the writing of the manuscript. All authors read and approved the manuscript.

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