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ABSTRACT

In vitro plant embryogenesis and microcallus formation are systems which are required for plant regeneration, a process during which cell reprogramming and proliferation are critical. These systems offer many advantages in breeding programmes, such as doubled-haploid production, clonal propagation of selected genotypes, and recovery of successfully gene-edited or transformed plants. However, the low proportion of reprogrammed cells in many plant species makes these processes highly inefficient. Here we report a new strategy to improve *in vitro* plant cell reprogramming using small molecule inhibitors of mammalian leucine rich repeat kinase 2 (LRRK2), which are used in pharmaceutical applications for cell reprogramming, but never used in plants before. LRRK2 inhibitors increased *in vitro* embryo production in three different systems and species, microspore embryogenesis of oilseed rape and barley, and somatic embryogenesis in cork oak. These inhibitors also promoted plant cell reprogramming and proliferation in Arabidopsis protoplast cultures. The benzothiazole derivative JZ1.24, a representative compound of the tested molecules, modified the expression of the brassinosteroid (BR)-related genes *BIN2, CPD*, and *BAS1*, correlating with an activation of BR signaling. Additionally, the LRRK2 inhibitor JZ1.24 induced the expression of the embryogenesis marker gene *SERK1-like*. The results suggest that the use of small molecules from the pharmaceutical field could be extended to promote *in vitro* reprogramming of plant cells towards embryogenesis or microcallus formation in a wider range of plant species and *in vitro* systems. This technological innovation would help to develop new strategies to improve the efficiency of *in vitro* plant regeneration, a major bottleneck in plant breeding.

1. Introduction

Plant *in vitro* culture techniques are critical to many modern approaches to crop improvement. They are essential players in accelerating breeding through several technologies: the use of doubled-haploids produced by microspore embryogenesis, the clonal propagation of elite genotypes, and the conversion of gene editing or transformation events into plants with improved traits. *In vitro* plant regeneration, through embryogenesis and organogenesis, is based on the induction of cellular reprogramming of different types of somatic cells, which either directly or indirectly (via proliferating cell masses or microcalli) give rise to vegetative organs or embryos and finally to plants (Germanà and Lambardi, 2016). Despite the huge applications of this technology, it is still highly, or even completely inefficient for many plant species of economic interest.

Improved regeneration of transgenic crop plants has been documented with the overexpression of morphogenic genes in certain species (Gordon-Kamm et al., 2019; Che et al., 2022). However, this approach is feasible only in a limited number of species where transformation techniques are established, severely restricting its applicability to commercially important genotypes. Furthermore, genetic transformation and genome editing remain challenging for many key crops due to their poor regeneration capacity in tissue culture. Thus, there is an urgent demand for efficient technologies to enhance *in vitro* plant

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regeneration and propagation, thereby advancing breeding efforts.

Therefore, new strategies are needed to identify new chemical promoters for *in vitro* plant cell reprogramming and regeneration. For years, the technology has been developed and fine-tuned by empirical approaches testing different types of culture media, growth conditions, donor plant ages, explant types, and treatments with a handful of phytohormones (Jain and Gupta, 2018; Loyola-Vargas and Ochoa-Alejo, 2018; Neumann et al., 2009; Maluszynski et al., 2003).

Despite the rapid growth of chemical biology approaches in biomedicine, the identification of physiologically active compounds in chemical libraries has not been efficiently explored in plant biotechnology yet. In the case of plant cell reprogramming and somatic embryogenesis, synthetic small molecules from chemical libraries have not been tested, apart from a few pioneering studies. A recent report from our group showed that several types of small molecules with reprogramming effects on mammalian cells, inhibitors of mammalian glycogen synthase kinase 3 (GSK3), can also improve plant cell reprogramming and *in vitro* somatic embryo production yield in several crop and forest species (Berenguer et al., 2021). Physiological and molecular analyses showed that these small molecules inhibited GSK3-like activity in oilseed rape, activated the brassinosteroid signalling pathway, and induced the expression of embryogenesis genes (Berenguer et al., 2021), ultimately promoting somatic embryogenesis.

Small molecule inhibitors are also known for the leucine rich repeat kinase 2 (LRRK2), an enigmatic enzyme and a relevant target in Parkinson's disease. It is a member of the Ras-like GTPase (ROCO) superfamily with 6 independent domains, including a kinase domain. Although its physiological roles in mammals are not yet well understood, LRRK2 has been implicated in adult neurogenesis through the Wnt signalling pathway (Berwick and Harvey, 2013). Interestingly, several LRRK2 inhibitors having a benzothiazole scaffold have been shown to modulate the Wnt/β-catenin signalling pathway, promote proliferation of neural progenitors and drive their differentiation towards neuronal and oligodendrocytic cell fates (Zaldivar-Diez et al., 2020). In plants, orthologues of LRRK2 have not been identified.

Growing evidence suggests that stem cells exhibit similar behaviors in both animal and plant systems (Olariu et al., 2017; Wittmer and Heidstra, 2024). In the quest for novel chemical agents to enhance plant cell reprogramming, LRRK2 inhibitors could be considered as promising candidates for promoting somatic embryogenesis in plants due to their demonstrated efficacy in stimulating cell proliferation and differentiation in mammalian models.

Here, we present the use of synthetic small molecule inhibitors targeting mammalian LRRK2, which belong to two distinct chemical classes. These inhibitors have not previously been employed in plants and were assessed across various species and *in vitro* systems. We examined their potential to enhance somatic embryogenesis in *Brassica napus* (oilseed rape), *Hordeum vulgare* (barley), and *Quercus suber* (cork oak). Additionally, their effects on promoting protoplast proliferation and microcallus formation were investigated in Arabidopsis.

2. Material and methods

2.1. Microspore embryogenesis of Brassica napus, through isolated microspore culture

Brassica napus L. cv. 'Topas' line DH407 plants were used as donor plants. Germination and growth of oilseed rapes were carried out in a growth chamber (Sanyo MLR-351-H) in pots containing a mixture of organic substrate and vermiculite (2:1, v/v) under the following conditions: relative humidity 60 %, 15 °C under a long-day photoperiod of 16 h light, 10 °C under 8 h of darkness, and watering three times per week, without external fertilization.

Flower buds at the vacuolated microspore stage, known for being highly responsive to microspore induction, were chosen for microspore culture following the methodology outlined by Prem et al. (2012). The buds underwent sterilization for 20 min using a 2% (v/v) bleach solution containing 2% active chlorine, followed by five rinses with sterile distilled water. Approximately 20 buds were crushed in 5 ml of cold NLN-13 medium (Lichter, 1982) with 13% (w/v) sucrose, using a pre-chilled mortar and pestle. The resulting suspension was filtered through a 48 μm nylon mesh, and the filtrate was collected into a 15 ml Falcon centrifuge tube. The crushed material was washed with an additional 5 ml of NLN-13 to bring the total volume to 10 ml, then centrifuged at 1100 rpm for 5 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 10 ml of cold NLN-13 medium. This centrifugation and resuspension process was repeated three times to thoroughly wash the microspores. The final pellet was resuspended in NLN-13 medium, and the cell density was adjusted to 10 000 cells per ml. The prepared cell suspension was dispensed into 90-mm Petri dishes at a volume of 10 ml per dish and cultured in darkness. To induce embryogenesis, the microspore cultures underwent an *in vitro* stress treatment at 32 °C. About four days after the start of the culture, the microspores began to reprogram and form proembryos, marking the onset of embryogenesis. When globular or heart-shaped embryos appeared, 20 days into the process, the cultures were transferred to 25 °C and agitated on an orbital shaker at 60 rpm in the dark. After roughly 30 days, the proembryos developed further into cotyledonary embryos.

2.2. Microspore embryogenesis of Hordeum vulgare, through isolated microspore culture

Hordeum vulgare L. cv. Igri plants were used as donor plants. Seeds were vernalised in soil for 1 month at 4 °C, and then transferred to a plant growth chamber at 18 °C for one month for germination and growth, with photoperiod of 16 h light and moderate watering every two days. Finally, the plants were transferred to a greenhouse at 18 °C under natural photoperiod conditions.

Spikelets containing microspores at the vacuolated microspore stage were collected and surface-sterilized by immersing them in a 5% (v/v) bleach solution with 5% active chlorine for 20 min, followed by four rinses with sterile distilled water. The culture preparation was carried out as described by Rodríguez-Serrano et al. (2012). The sterilized spikes underwent a pre-treatment at 4 °C for 21–24 days as a stress treatment to induce microspore embryogenesis. The microspores were isolated by blending the spikes in 20 ml of pre-cooled 0.4 M mannitol at 4 °C, using a Waring Blender pre-chilled to −20 °C. The extract was then filtered through a 100 μm nylon mesh into a beaker pre-cooled to −20 °C. The resulting microspore suspension was transferred into a 50 ml tube and centrifuged at 800 rpm for 10 min at 4 °C. After discarding the supernatant, the pellet was resuspended in 4 ml of pre-cooled 0.55 M maltose and transferred into a 15 ml Falcon tube. Subsequently, 1.5 ml of 0.4 M mannitol solution was carefully added without mixing. Following gradient centrifugation at 800 rpm for 10 min at 4 °C, the interphase band containing an almost pure population of vacuolated microspores was resuspended in a 0.4 M mannitol solution, resulting in a final volume of 10 ml. The microspores were counted using a Neubauer chamber, and the pelleted microspores were diluted in an appropriate volume of KBP medium (Kumlehn et al., 2006) to achieve a cell density of 1.1 x 10^5 cells per ml. They were then plated in 30 mm Petri dishes at a volume of 1 ml per plate. The microspore cultures were incubated at 25 °C in the dark, and after 4 days of cultivation, some microspores reprogrammed and produced multicellular structures or proembryos. These proembryos further developed into coleoptylar and mature embryos, which were observed 30 days later.

2.3. Small molecule inhibitors of LRRK2

Four different LRRK2 inhibitors with two different heterocyclic scaffolds, benzothiazole and indolinone, belonging to our in-house chemical library (MBC library, Ginex et al., 2023) were tested. These

four inhibitors were the benzothiazole-based JZ1.24 (mol. wt 418 Da), JZ1.3 (mol. wt 353 Da) and JZ1.6 (mol. wt 357 Da) (Zaldivar-Diez et al., 2020), together with the indolinone derivative IGS4.75 (mol. wt 231 Da) (Salado et al., 2017) (Fig. 1). The inhibitory potency for each compound, represented by the IC_{50} value, is 0.51 μM for JZ1.24, 0.48 μM for JZ1.3, 0.17 μM for JZ1.6 and 1.70 μM for IGS4.75.

No special safety requirements are needed to handle these compounds other than general safety precautions when handling chemicals.

2.4. Treatments with small molecule inhibitors on microspore embryogenesis cultures of B. napus and H. vulgare

The LRRK2 inhibitors were added to the microspore liquid culture media by using stock solutions of 10 mM in dimethyl sulfoxide (DMSO). Appropriate volumes of stock solutions of the drugs were added to the culture media to obtain the selected working concentrations of the inhibitors, keeping the DMSO concentration below 0.2 % (v/v). In *B. napus* microspore cultures, the 4 inhibitors were tested at 4 different concentrations, ranging from 0.5 to 5 μM. In *H. vulgare* microspore cultures, one selected inhibitor was tested at three selected concentrations (1, 2.5 and 5 μM).

Compounds were added from culture initiation and kept until the end of culture. Their effect on embryogenesis efficiency was assessed after 4 days of treatment, when proembryos were produced. Embryo production was recorded after 30 days from the start of culture initiation. Mock-treated plates, with the same volume of DMSO and without the inhibitors, were maintained, as controls.

2.5. Evaluation of embryogenesis induction efficiency and embryo production in microspore cultures of B. napus and H. vulgare

The efficiency of embryogenesis induction was quantified in untreated and treated cultures by the number of proembryos formed (considered the first sign of embryogenesis initiation), as previously described (Berenguer et al., 2017). Proembryos were easily identified under inverted microscope in 4-day culture plates as round-shaped multicellular structures of larger size and density than microspores, still surrounded by the exine (special microspore wall). Proembryos produced from untreated and treated microspores after 4 days, and in the case of barley, also embryos developed after 30 days were quantified from images taken under a stereomicroscope (LEICAMZ16F) by using image analysis tools (Adobe Photoshop CS5.1). The mean percentage of proembryos and the mean number of embryos per plate were obtained from three independent experiments/cultures per each *in vitro* system and treatment. with at least three different culture plates in each experiment. A minimum of 1000 proembryos were counted for each treatment and plant species. Results on proembryos were expressed as percentages (percent change) and referred to the mean percentage of proembryos in control cultures, normalised to 100%. Each individual treatment (a compound at a specific concentration) was compared with the control; significant differences between each pair of conditions (control *vs* treatment) were determined by applying Student's-*t*-test ($P \leq 0.05$). In the experiments in B. napus, where four different concentrations were tested for each compound, differences among concentrations were tested by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test at $P \leq 0.05$.

2.6. Evaluation of germination capacity of microspore-derived embryos

The embryos produced after 30 days were analyzed. Embryo germination assays were performed to evaluate the quality of the embryos produced in the presence of the inhibitors. Oilseed rape microspore cotyledonary embryos originated from control and treated-cultures were used for *in vitro* embryo germination and conversion to plantlets as previously described (Prem et al., 2012). The 30-day-old dicotyledonous embryos were air-dried on sterile filter paper and germinated in MS medium (Murashige and Skoog, 1962) containing MS vitamins, sucrose 2 % (w/v) and gelled with 7 g l^{-1} bacteriological agar (w/v). Microspore-derived embryos were incubated for 15–20 days at 18 °C in the dark until radicle and plumule activation, and quantified as the percentage of embryos showing normal growth, similar to zygotic embryo germination. To achieve plantlet conversion, embryos that had germinated were transferred to growth conditions of 25 °C under a 16-h photoperiod for 10 days. Subsequently, they were transferred to tubes for continued growth and ultimately acclimated to external conditions in pots, following the protocol described by Prem et al. (2012).

2.7. Somatic embryogenesis of Quercus suber, through immature zygotic embryo culture

Immature pollinated acorns were collected from *Quercus suber* L. (cork oak) trees in the countryside (El Pardo region, Madrid, Spain) during the fruit development period (late August and September). Immature acorns were selected at the stage most responsive to somatic embryogenesis induction, corresponding to immature zygotic embryos at the early cotyledonary stage. Immature acorns are small, about 1 cm diameter, and green in colour. After collection, immature acorns were kept at 4 °C for one week before starting *in vitro* culture. Then, immature acorns were sterilized by immersion in 70% (v/v) ethanol for 30 s and in 2% (v/v) sodium hypochlorite for 20 min, followed by 3 rinses in sterile distilled water of 10 min each. Immature zygotic embryos were then carefully dissected from the acorns by removing the surrounding tissues using a scalpel and forceps, and cultured in induction media, according to the updated protocol described by Testillano et al. (2018). This media contains Sommer macronutrients, MS micronutrients, MS vitamins, 0.5 mg l^{-1} glutamine, 30 g l^{-1} sucrose, 8 g l^{-1} bacteriological agar, and 0.5

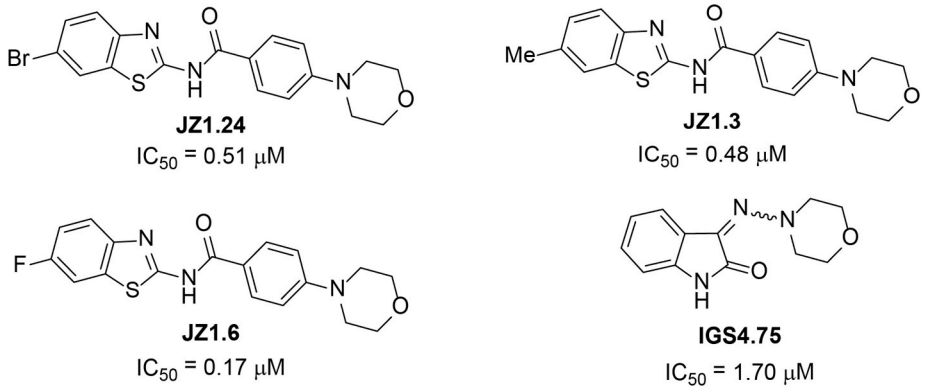

Fig. 1. Chemical structures of the LRRK2 inhibitors. The four small molecules are named JZ1.24, JZ1.3, JZ1.6, and IGS4.75. The IC50 value indicates the inhibitory potency of the compound, which is the concentration required to inhibit, *in vitro*, 50% of the human LRRK2 enzymatic activity.

mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich, Saint Louis, MO, United States). Culture conditions were 16/8 h light/darkness at 25 °C. After one month, the explants were transferred to solid proliferation medium, with the same composition as the induction medium but without 2,4-D and supplemented with L-glutamine. During the next weeks of culture cell reprogramming occurs in some responsive cells which initiated the embryogenesis pathway, producing small embryogenic masses and embryos (Testillano et al., 2018). Cultures were transferred monthly to the same fresh medium, and somatic embryogenesis cultures continued their development and multiplied, producing new proliferative masses and somatic embryos.

2.8. Treatment with small molecule inhibitors and evaluation of the effect over somatic embryogenesis of Q. suber

In cork oak, embryogenic masses were treated with a selected inhibitor at two selected concentrations (50 and 100 μM). The inhibitor was added from the beginning of the culture. In parallel, mock-control cultures in which embryogenic masses were cultured without the inhibitor were established. After 30 days of treatment, embryogenic cultures were transferred to a recovery medium consisting of a normal proliferation medium without the inhibitor.

The efficiency of embryogenesis induction in control and treated cultures was quantified by the number of cotyledonary embryos produced after 30 days of treatment (culture medium containing the inhibitor) followed by 30 days of recovery (culture medium without inhibitor). Embryo production was determined by quantifying the number of cotyledonary embryos produced per gram of embryogenic masses at culture initiation.

Three independent experiments/cultures were conducted for each treatment, with at least two plates analyzed per experiment, each containing 5 embryogenic masses. Each individual treatment (the compound at a specific concentration) was compared with the control; significant differences between each pair of conditions (control *vs* treatment) were determined by applying Student's-t test ($P \leq 0.05$).

2.9. Gene expression analyses by quantitative real-time (RT-qPCR)

In *B. napus*, expression of brassinosteroid related genes was analyzed. Untreated microspore embryogenesis cultures and JZ1.24-treated cultures were selected for expression analyses. The sequences of the BR pathway gene *BRASSINOSTEROID-INSENSITIVE2* (*BnBIN2*), the BR biosynthesis gene *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* (*BnCPD*), and the BR inactivation gene *PHYB ACTIVATION-TAGGED SUPPRESSOR1* (*BnBAS1*), were selected from the *Brassica rapa* database (www.brassicadb.cn). The sequence of the primers used are indicated in Supplementary Table S1.

Total RNA was extracted from *in vitro* samples using the RNeasy® Plant Mini Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. To remove contaminating DNA, RNA was treated with RNase-free DNase using the Turbo DNA-freeTM Kit (Ambion, Life Technologies, Carlsbad, CA, USA), according to the supplier's protocol. cDNAs were synthesized from 1.5 μg of RNA using Superscript™ II Reverse Transcriptase (Invitrogen Life Technologies). RT-qPCR analyses were performed using the FastStart DNA Green Master (Roche Diagnostics) on the LightCycler®96 (Roche Diagnostics International Ltd.) under the following conditions: initial denaturation at 95 °C for 30 s, then 40 cycles of 5 s at 95 °C and 30 s at 58 °C. After each run, the specificity of amplification was checked by the acquisition of a dissociation curve by heating the samples from 65 °C to 95 °C. Data was analyzed with the LightCycler®96 software (v.1.1.0.1320) (Roche Diagnostics International Ltd.), and using the Livak calculation method (Livak and Schmittgen, 2001). As internal reference gene *HELICASE* (*AtHEL*) was used.

In *Q. suber,* expression of *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1-like (SERK1-like)* was analyzed in somatic embryogenesis cultures. Immature zygotic embryos and embryogenic masses from control cultures were selected for expression analysis. In addition, embryogenic masses treated with the inhibitor JZ1.24 for 30 days and untreated masses (controls) were also used. The sequence for *QsSERK1-like* was selected from the NCBI database (www.ncbi.nlm.nig.gov/genebank). The sequence of the primers used are indicated in Supplementary Table S1.

Total RNA from samples was purified with the NucleoSpin® RNA Plant (Macherey-Nagel, Düsen, Germany) according to the manufacturer's instructions. RAP buffer with 1% β-mercaptoethanol was used. Contaminated DNA was removed from the total RNA samples using the Turbo DNA-freeTM Kit. cDNAs were obtained from 300 ng aliquot of total RNA using the SuperscriptTM II Reverse Transcriptase. RT-qPCR conditions and data analyses were performed as described in *B. napus*. As internal reference gene *ACTIN* (*QsACT*) was used (Marum et al., 2012).

Oligonucleotides were designed with Primer 3 software and are listed in Supplementary Table S1. In *B. napus*, transcript levels were normalised to control culture samples, whereas in *Q. suber* data were normalised to immature zygotic embryos and to control cultures in the inhibitor study. Three biological and three technical replicates were performed. Differences between two conditions (control and treated cultures) were tested by Student's-t test, at $P \leq 0.05$.

2.10. Arabidopsis plant culture and protoplast isolation

Seeds from an *A. thaliana* line overexpressing histone 2B - yellow fluorescent protein fusion (H2B-YFP; Boisnard-Lorig et al., 2001) were surface-sterilized and sown on MS agar. After 24 h stratification, the plates were cultured vertically for 7 days at 22 °C under long day conditions (16 h light – 8 h dark). Prior to seedling harvest, the plates were stored vertically in the dark at 4 °C for 24 h. Protoplasts were isolated from hypocotyl and cotyledons including primary leaves according to Dovzhenko et al. (2003).

2.11. Arabidopsis protoplast immobilisation, small molecule inhibitors treatments and imaging

Protoplast density was determined by cell counting in a Fuchs-Rosenthal chamber. For immobilisation, protoplasts were mixed with equal volumes of 1.2 % (w/v) low melting agarose in protoplast buffer, pre-warmed at 40 °C and immediately pipetted into a 96-well plate (Ibidi, Germany), pre-warmed at 34 °C with 125 μl per well. The plate was immediately centrifuged (2 min, 30 g) and incubated at 4 °C for 5 min. 200 μl of culture medium was added to each well and the protoplasts were cultured at 22 °C in the dark (Kao and Michayluk, 1975). The growth medium consisted of macro- and microelements based on Kao and Michayluk (1975) supplemented with 2 μM 2,4-D and 1 μM thidiazurone. Protoplasts were immobilised with a final density of 15 000 protoplasts per well.

For small molecule treatments, chemicals were dissolved in DMSO and added to the growth medium. LRRK2 inhibitors IGS 4.75, JZ 1.3, JZ 1.6, and JZ 1.24 were tested at concentrations ranging from 100 nM to 1 μM. After 48 h, the culture medium was removed and replaced with fresh medium after two washes with culture medium.

An automated microscope (MORE1, Till I.D. GmbH, Munich, Germany) was used to analyse protoplast development and to detect YFP fluorescence. All experiments were performed with three biological replicates and significance was determined by a Student's t-test. Transillumination was recorded with 10x0.45 and 20x0.8 objectives (Zeiss); epifluorescence was recorded after excitation with single-mode diode laser excitation (iBeam smart, Toptica) with 550 nm (YFP)/green emission filter. Image acquisition was performed using the SIAM software (Till I.D. GmbH, Munich, Germany).

2.12. Image processing and data analysis of Arabidopsis protoplast cultures

Images were taken immediately after immobilisation and 7 days after immobilisation (DAIs). Image processing was performed using Fiji, an image processing package distributed by ImageJ2 (Schindelin et al., 2012; Rueden et al., 2017). Raw tile image stacks were reconstructed into full-well images using the stitching plugin (Preibisch et al., 2009). Image segmentation was performed using U-net (Falk et al., 2018). Quantification of proliferating cells was performed by exploiting shape differences of dividing or closely adjacent nuclei after applying EDM binary operations to epifluorescence images, compared to circular fluorescence signals of non-dividing mononuclear cells (Brocher, 2014). Corresponding cell shape parameters of proliferating microcalli were determined by selecting segmented transillumination images after filtering with processed epifluorescence images using the BioVoxxel binary feature extraction function. Proliferation events, identified by image analysis were quantified and values were normalised to the total cell number cultivated and expressed relative to the proliferation rate of control cells treated with equivalent volume of DMSO. All experiments were performed with three biological replicates. Differences among treatments were tested by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test at $P \leq 0.05$.

3. Results

In an attempt to identify novel strategies to promote plant cell reprogramming, we have selected compounds with reported activity as promoters of cell proliferation and differentiation from the Medicinal and Biological Chemistry library (MBC, Ginex et al., 2023).

In our study, we have tested a total of four small molecule inhibitors of LRRK2: JZ1.24, JZ1.3, JZ1.6 and IGS4.75, with different molecular structures (Fig. 1). These compounds have shown LRRK2 inhibitory activity with an IC_{50} value in the micromolar range (Salado et al., 2017; Zaldivar-Diez et al., 2020). These four molecules, featuring a benzothiazole core, have been demonstrated to stimulate the proliferation of neural progenitor cells, and steer their differentiation into neuronal and oligodendrocyte lineages (Zaldivar-Diez et al., 2020); consequently, we investigated whether these compounds could similarly promote plant cell proliferation and embryogenesis.

Four different *in vitro* culture systems, well established from previous literature, were used to test the new compounds (Table 1). First, the four inhibitors were tested in assays with oilseed rape microspore cultures, with 3–4 different concentrations tested for each compound. After evaluating the effects of the inhibitors on microspore reprogramming and embryogenesis initiation efficiency in oilseed rape microspore embryogenesis, one compound was selected and tested at one concentration in the *in vitro* embryogenesis systems of barley and cork oak. In parallel, the four inhibitors were tested in Arabidopsis protoplast cultures at concentrations ranging from 100 nM to 1 μM.

Table 1
Plant species and *in vitro* culture systems used to test the effects of the mammalian LRRK2 inhibitors.

Plant species	Process	Type of culture	References
Brassica napus (oilseed rape)	Microspore embryogenesis	Isolated microspores in liquid medium	Prem et al. (2012)
Hordeum vulgare (barley)	Microspore embryogenesis	Isolated microspores in liquid medium	(Rodríguez-Serrano et al., 2012)
Quercus suber (cork oak)	Somatic embryogenesis	Immature zygotic embryos on solid medium	Testillano et al. (2018)
Arabidopsis thaliana	Microcallus formation	Agarose-embedded protoplasts in liquid medium	Dovzhenko et al. (2003)

3.1. Effect of LRRK2 inhibitors on microspore embryogenesis of Brassica napus

In oilseed rape microspore embryogenesis cultures, in response to the inductive treatment, isolated microspores at the responsive developmental stage of vacuolated microspore (Fig. 2A) divide to produce multicellular structures or proembryos (Fig. 2B), that are still confined within the microspore cell wall (exine). Such structures are considered to be the first sign of embryogenesis initiation and can be found after 4 days in culture. Globular and heart-shaped embryos can be observed at 20 days after culture initiation. Normally, after 30 days in culture, the embryos are fully developed and form cotyledonary embryos (Fig. 2C–D).

The efficiency of embryogenesis induction was evaluated in control cultures and cultures treated with the inhibitors, at different concentrations, ranging from 0.5 to 5 μM. The results showed that all the inhibitors tested resulted in an increase in the efficiency of embryogenesis induction in the range of 23–30 % when applied at their optimal concentration (Fig. 2E). Based on these results, a representative small molecule was selected for further analysis and testing in other *in vitro* embryogenesis systems. The molecule selected was JZ1.24, which showed one of the best results, with 27.5% increase in embryogenesis induction efficiency at 2.5 μM.

Embryo production and germination capacity of the embryos produced in microspore cultures treated with the inhibitors were evaluated. Cotyledonary embryos produced in JZ1.24-treated cultures after 30 days were anatomically similar to those developed in control cultures, while the embryo production was higher in treated cultures (Fig. 2F and G). For germination assays, fully developed cotyledonary embryos from control and treated cultures were desiccated and cultured under germination conditions (Prem et el. 2012). The results showed that the germination of the embryos was not affected by the treatment with the inhibitor. These embryos produced roots and hypocotyls similar to and in the same proportion as embryos from control cultures (Fig. 2H). Regardless of the application of inhibitors during *in vitro* embryogenesis, all embryos that germinated successfully developed into *in vitro* plantlets. These plantlets further acclimatized and progressed into mature plants, reaching the flowering stage and exhibiting phenotypes similar to those of donor plants (data not shown), consistent with findings reported for microspore-derived embryos of *B. napus* (Prem et al., 2012).

3.2. Effect of LRRK2 inhibitor on expression of brassinosteroid pathway genes during microspore embryogenesis initiation of B. napus

Previous studies have demonstrated that the endogenous brassinosteroids (BR) pathway is activated during the initiation of microspore embryogenesis (Berenguer et al., 2021), being GSK3-BIN2 a negative regulator of the BR signaling (Nolan et al., 2020). On the other hand, some studies indicated that mammalian LRRK2 interacts with GSK3 (Kawakami et al., 2013; Kofoed et al., 2020), suggesting that LRRK2 inhibitors could have a negative impact on GSK3. Therefore, we investigated the potential impact of the LRRK2 inhibitor JZ1.24 on the expression of BR pathway genes during microspore embryogenesis initiation of *B. napus*. We compared the expression levels of key components involved in BR signaling, biosynthesis, and catabolism in both control cultures and cultures treated with the inhibitor JZ1.24, specifically at the proembryo stage, after embryogenesis induction. Our analysis focused on BIN2, the principal negative regulator of BR signaling, which is a GSK3 kinase that becomes inhibited in the presence of BR, thereby triggering the BR signaling cascade (Nolan et al., 2020). The findings indicated that *BnBIN2* expression was reduced in JZ1.24-treated cultures (Fig. 3), implying that the compound may activate the BR signaling pathway. Furthermore, we examined the expression of *CPD* and *BAS1*, essential elements of the BR biosynthesis and catabolic pathways, respectively, in both control and JZ1.24-treated cultures. RT-qPCR assays revealed a decrease in the expression of the

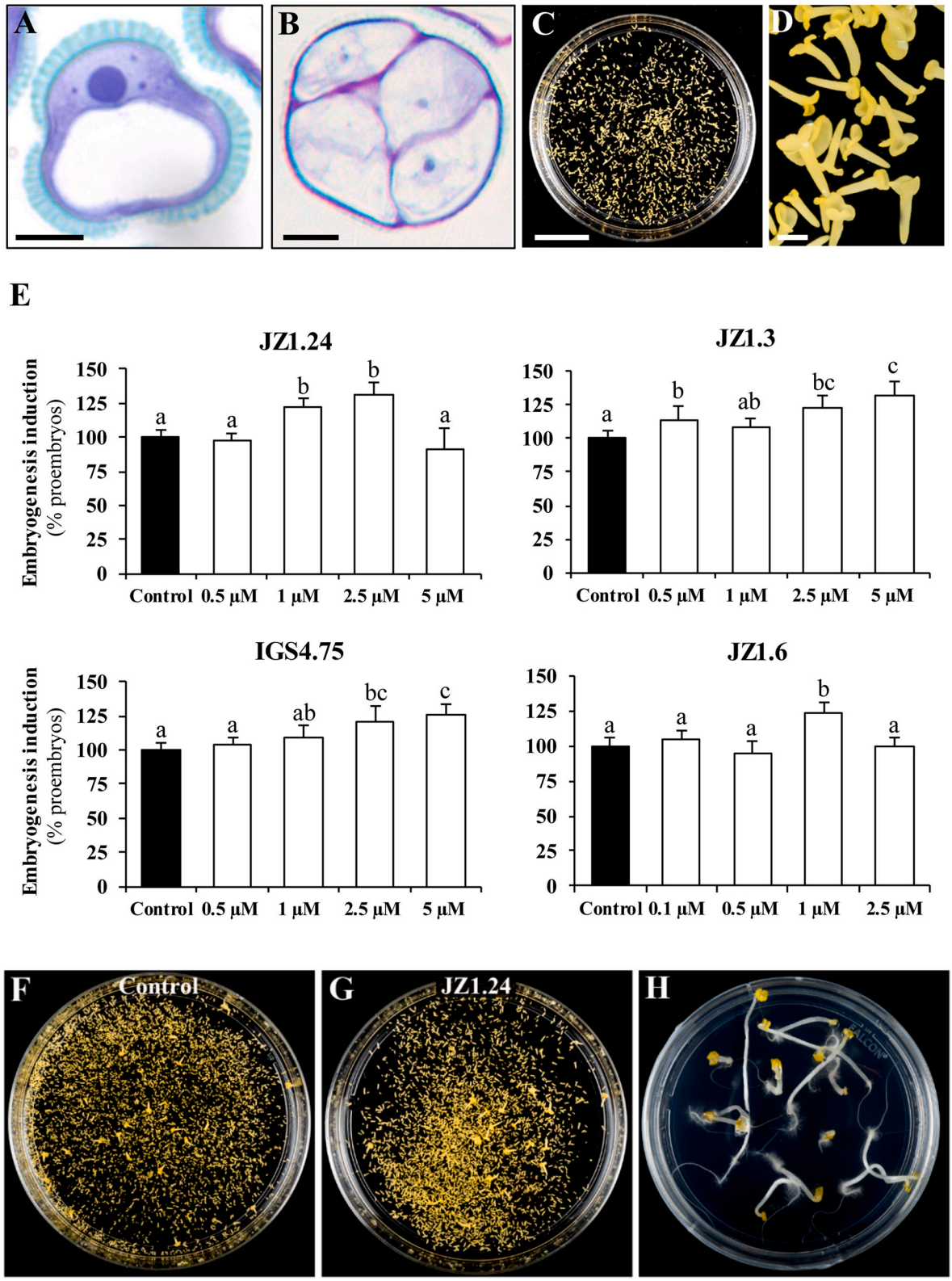

Fig. 2. *M*icrospore embryogenesis in *B. napus*, effects of LRRK2 inhibitors over embryogenesis induction, embryo production and germination capacity. (A–D) Main stages of microspore embryogenesis in *B. napus*. (A, B) Toluidine blue-stained sections of isolated vacuolated microspore (A, culture initiation) and proembryo (B, embryogenesis initiation). (C, D) Cotyledonary embryos; panoramic view of a plate (C) and detail at higher magnification. (D). (E) Effects of LRRK2 inhibitors over embryogenesis induction efficiency. Columns represent percent change of proembryos after 4 days from culture initiation, and refer to the mean percentage of proembryos in control cultures (normalised to 100%). Bars indicate the standard error of the mean, and different letters indicate significant differences according to ANOVA and Tukey's test at $P \leq 0.05$. (F, G) Cultures after 30 days, showing production of embryos in control (F) and JZ1.24-treated cultures (G). (H) Germinating embryos from JZ1.24-treated culture showing well-developed roots and hypocotyls in most embryos. Bars represent: (A, B) 10 µm; (C, F-H) 2 cm; (D) 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

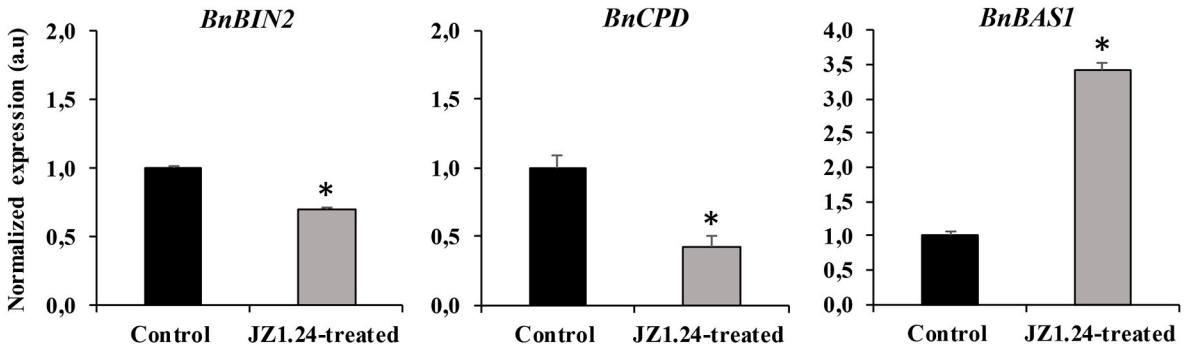

Fig. 3. Effect LRRK2 inhibitor JZ1.24 on gene expression profiles of brassinosteroid related genes during microspore embryogenesis initiation of *B. napus*. Expression of *BnBIN2*, *BnCPD* and *BnBAS1* in untreated and JZ1.24-treated microspore cultures of *B. napus* at the stage of embryogenesis initiation (proembryo formation, 4 days). Bars indicate the standard error of the mean, and asterisks indicate significant differences ($P \leq 0.05$) after Student's-*t*-test.

BnCPD biosynthesis gene, while the *BnBAS1* catabolic gene expression increased, in treated cultures (Fig. 3). These expression profiles aligned with the activation of the BR pathway, characterized by decrease in the transcription of the feedback-regulated BR biosynthetic gene *CPD* and an increase in the BR-inducible gene *BAS1* (Nolan et al., 2020). Therefore, these observations suggested that JZ1.24 may enhance BR signaling.

3.3. Effect of LRRK2 inhibitor on microspore embryogenesis of Hordeum vulgare

After induction, barley microspores (Fig. 4A) reprogrammed to produce multicellular structures or proembryos (Fig. 4B) which could be seen after 4 days in culture. The proembryos developed further to produce transitional, coleoptylar and mature embryos (Fig. 4C–D), which were observed after 30 days.

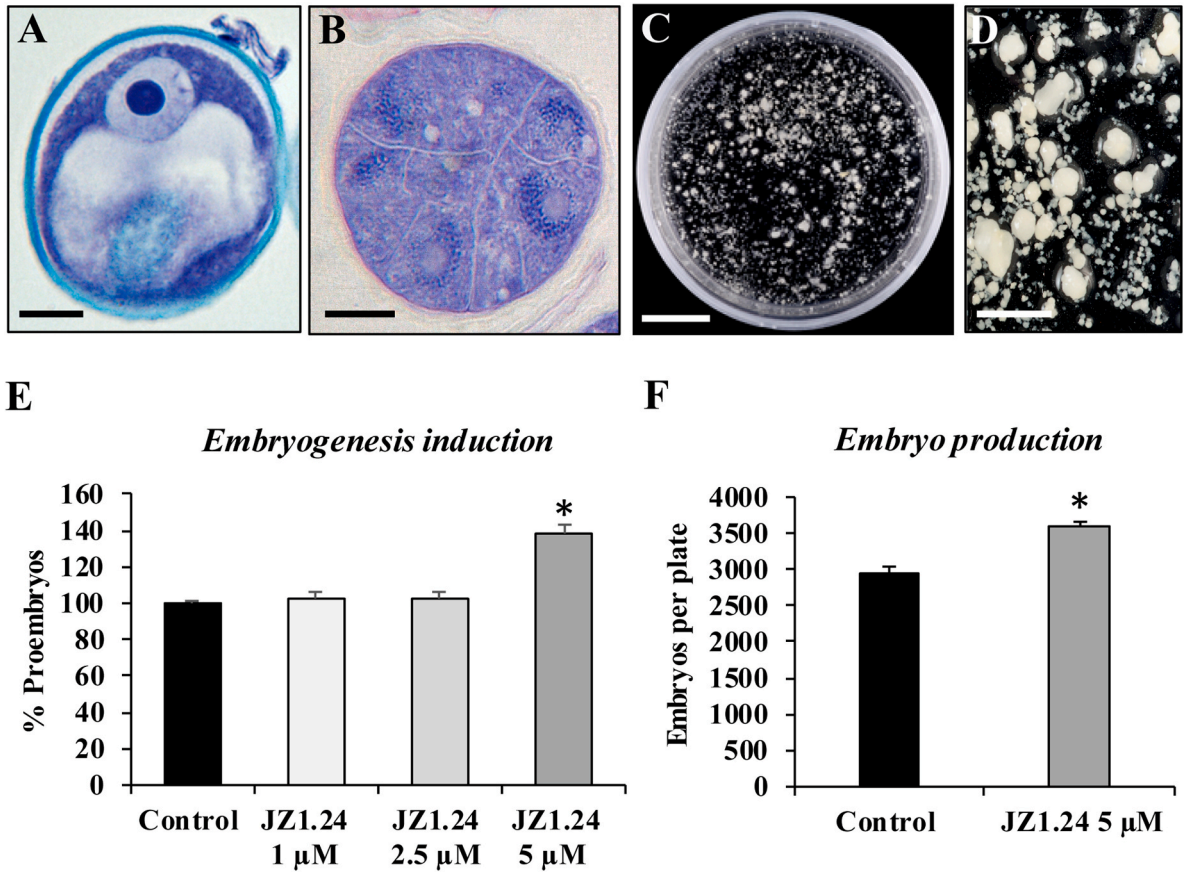

Fig. 4. *Microspore embryogenesis in H. vulgare,* effect of LRRK2 inhibitor JZ1.24 on embryogenesis induction and embryo production. (A–D) Main stages of microspore embryogenesis in *H. vulgare*. (A, B) Toluidine blue-stained sections of isolated vacuolated microspore (A, culture initiation) and proembryo (B, embryogenesis initiation). (C, D) Transitional and coleoptilar embryos; panoramic view of a plate (C) and detail at higher magnification (D). (E) Effect of JZ1.24 over embryogenesis induction efficiency. Columns indicate percent change of proembryos produced after 4 days from culture initiation in control (untreated) and treated cultures. Values have been normalised to the control culture (100%). (F) Effect of JZ1.24 over embryo production. Columns indicate mean number of embryos per plate formed at 30 days in control (untreated) and treated cultures. Bars indicate the standard error of the mean, and asterisks indicates significant differences ($P \leq 0.05$) after Student's-*t*-test. Bars represent: (A, B) 10 µm; (C) 2 cm; (D) 500 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The selected inhibitor, JZ1.24, was applied to microspore embryogenesis cultures of *H. vulgare* at 1, 2.5 and 5 μM concentrations. Quantification of proembryos formed after 4 days showed that treatments with the inhibitor at a concentration of 5 μM improved the efficiency of embryogenesis induction, with an increase in proembryo formation of 48 % in JZ1.24-treated cultures (Fig. 4E).

Untreated and treated cultures were further developed and the total number of embryos produced per plate after 30 days was quantified. Microspore cultures treated with the inhibitor produced more embryos than control cultures, with an increase of 22 % in JZ1.24-treated cultures (Fig. 4F).

3.4. Effect of LRRK2 inhibitor on somatic embryogenesis of Quercus suber

To evaluate the possibility of extending the findings from *B. napus* and *H. vulgare* to more distant species and processes, the selected inhibitor JZ1.24, was applied to a forest woody species, *Q. suber*, in which somatic embryogenesis was established from immature zygotic embryos, an *in vitro* system consisting of two-stage cultures in solid media. Immature zygotic embryos were obtained from young acorns (Fig. 5A) and placed in culture. After the induction of embryogenesis, proembryogenic masses were produced and protruded from different parts of the initial explants, the immature zygotic embryo (Fig. 5B); they continued to proliferate and produced new embryogenic masses, which in turn gave rise to new embryos (Fig. 5C), which by recurrent and secondary embryogenesis, developed into ivory-coloured cotyledonary embryos (Fig. 5D–E).

The inhibitor treatment was applied at two distinct concentrations. In gelled media, compounds exhibit reduced diffusion and availability to explant cells compared to liquid media and isolated cells. As a result, the concentrations used for cork oak explants in gelled medium were 10–20 times higher than those used for isolated microspore cultures in liquid medium. Specifically, the inhibitor was applied at concentrations of 50 and 100 μM. These concentrations fall within the range reported in previous studies using small molecules in this *in vitro* system (Berenguer et al., 2021; Carneros et al., 2024). The evaluation of the effects of the compound on embryogenesis efficiency was assessed by quantifying the number of cotyledonary embryos produced in control and treated cultures. The results showed that treatment with the inhibitor increased the efficiency of embryogenesis induction and resulted in 3.4 times higher somatic embryo production (Fig. 5F).

The SERK1-gene has been described to play a positive role in the

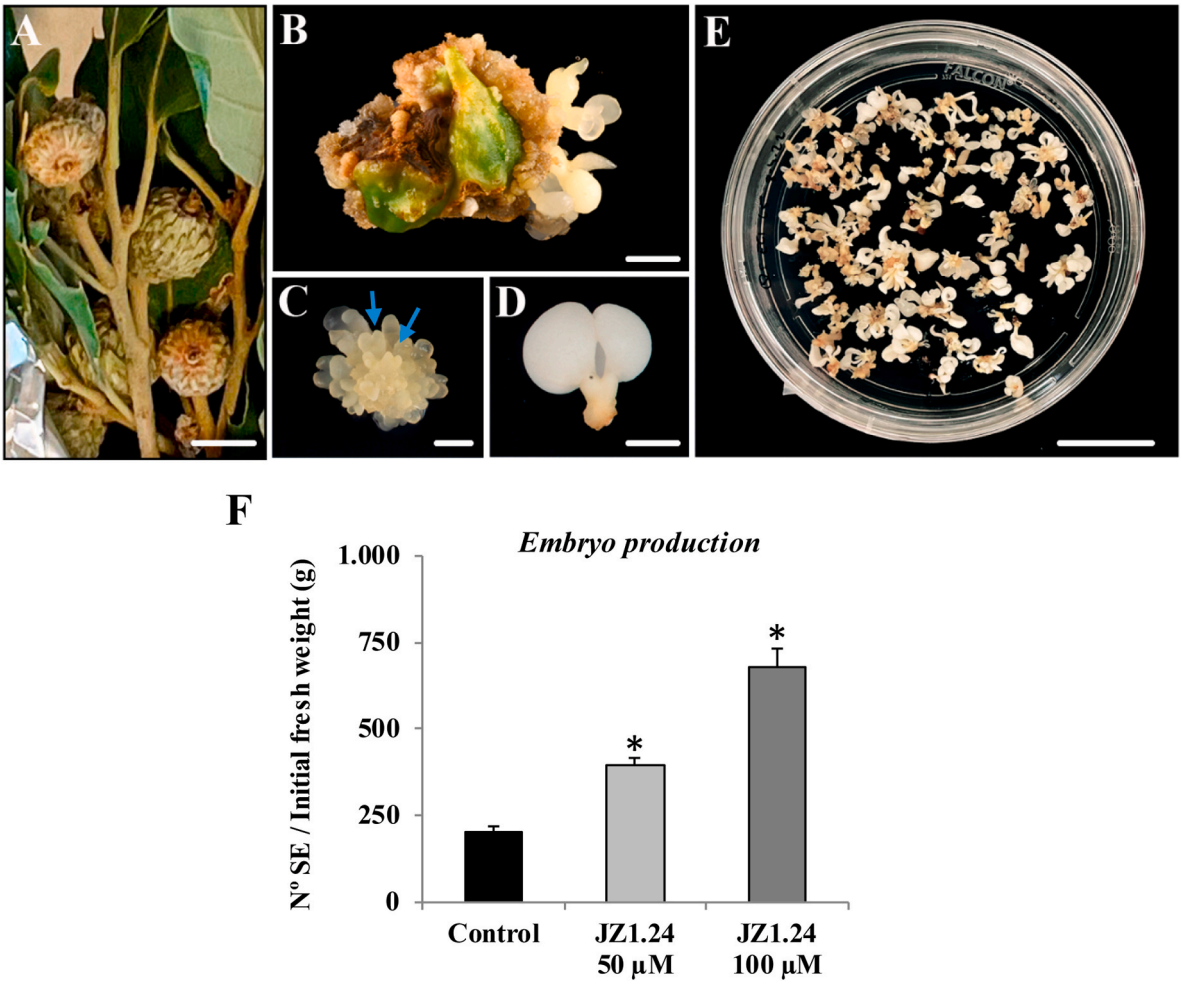

Fig. 5. Somatic embryogenesis in *Q. suber* and effect of LRRK2 inhibitor JZ1.24 over embryo production. (A–E) Main stages of somatic embryogenesis in *Q. suber*, (A) Acorns containing immature zygotic embryos (initial explant for somatic embryogenesis induction). (B) Embryogenic masses and somatic embryos arising from the immature zygotic embryo. (C) Heart-shaped embryos (arrows) differentiating from embryogenic mass. (D) Mature cotyledonary embryo. (E) Panoramic view of a culture plate showing different structures corresponding to various developmental stages of cork oak somatic mbryogenesis process. (F) Quantification of the effect of JZ1.24 over somatic embryo production, bars represent the mean number of embryos per gram of initial explant, in control (untreated) and treated cultures after 30-day treatment followed by 30 days on recovery conditions. Bars indicate the standard error of the mean, and asterisk indicates significant differences between the control and each individual treatment (each concentration), by applying Student's-*t*-test ($P \leq 0.05$) to compare the two conditions (control and treatment). Bars represent: (A) 0.5 cm; (B) 500 μm; (C, D) 100 μm; (D) 1 cm.

initiation of the embryogenesis response as well as in the acquisition of embryogenic competence in embryogenic cultures (Schmidt et al., 1997). Our results indicated that in control somatic embryogenesis cultures, the expression of QsSERK1-like was twice as high in embryogenic masses compared to immature zygotic embryos following the induction of embryogenesis (Fig. 6A). To gain deeper insights into the effect of the LRRK2 inhibitor JZ1.24 on embryogenic cork oak cultures, we analyzed the expression profiles of the embryogenesis-specific gene in treated cultures. Embryogenic masses treated with JZ1.24 showed a significant induction of QsSERK1-like compared to untreated masses (Fig. 6B). This result indicated that the treatment with the inhibitor increased the expression of the early embryogenesis marker gene.

3.5. *Effect of LRRK2 inhibitors on the efficiency of mesophyll protoplast microcallus induction in Arabidopsis thaliana*

LRRK2 inhibitors were tested for their ability to increase the proliferation rate of Arabidopsis protoplasts isolated from leaves. An Arabidopsis line overexpressing H2B-YFP was used as a suitable system to monitor proliferating protoplasts identified by the number of fluorescent nuclei. Immobilised protoplasts were overlaid with growth medium supplemented with inhibitors at concentrations from 100 nM to 1 μM, since higher concentrations were toxic for this *in vitro* system. Microscopic images were taken every 24 h during 7 days after immobilisation, and proliferating microcalli were identified by the presence of multiple adjacent fluorescent nuclei visualised using the nuclear-encoded H2B-YFP marker (Fig. 7A). Analyses of the microscopic images indicated that treatments with the four molecules enhanced microcalli formation after 7 days in culture, in comparison with control cultures. An example of this positive effect of the inhibitors over proliferation is illustrated in Supplementary Fig. S1, which shows well overview images of control and JZ1.6-treated cultures.

Proliferation rates were quantified and normalised to the number of protoplasts immobilised in each well. The results showed an increased rate of microcalli formation with the four inhibitors; the increase, in comparison with control, was from 30 to 100 % for IGS 4.75, JZ 1.3, JZ 1.6 and JZ1.24 (Fig. 7B).

4. Discussion

Induction of somatic embryogenesis involves cell reprogramming through activation of different signalling pathways (Feher, 2015; Testillano, 2019; Ibáñez et al., 2020), while chemically distinct small molecules have been designed and shown to efficiently induce cell reprogramming in mammalian cells (Ma et al., 2017; Zaldivar-Diez et al., 2020). Since there is increasing evidence that stem cells in plants and animals behave in a similar manner (Olariu et al., 2017), we hypothesized that some of these new compounds that induce cell reprogramming in mammalian cells can efficiently enhance cell reprogramming in different plant *in vitro* systems and species.

In the present study we have identified some compounds, inhibitors of mammalian LRRK2, that promote plant cell reprogramming and proliferation in *in vitro* cultures of isolated cells like microspores and protoplasts as well as tissue explants. LRRK2 inhibitors enhanced embryo production *in vitro* across three distinct systems and species: microspore embryogenesis in oilseed rape and barley, and somatic embryogenesis in cork oak. These inhibitors also stimulated plant cell reprogramming and proliferation in Arabidopsis protoplast cultures. These LRKK2 inhibitors have been reported to promote neurogenesis as well as the *in vitro* proliferation of neurospheres, an *in vitro* model of cells derived from neural stem cells and neural progenitors (Zaldivar-Diez et al., 2020). In mammals, LRRK2 plays a key role in Wnt signalling, modulating the Wnt/β-catenin signalling pathway. Wnt signalling pathways play distinct roles during development, normal homeostasis and disease. The responses that result from the activation of this pathway control both, cell proliferation and differentiation (Teo and Kahn, 2010).

Protoplasts offer unique advantages in new plant breeding technologies and are increasingly used in current applications of CRISPR/Cas-based breeding, being a promising platform for DNA-free gene editing (Reed and Bargmann, 2021). However, the development of efficient protocols for protoplast proliferation and plant regeneration, following gene editing, is a technical barrier to the wider use of protoplasts in this field (Yue et al., 2021). The results of the present study have demonstrated the beneficial effects of small molecules with activity on LRRK2 inhibition, never before used in plant cell culture, on Arabidopsis protoplast proliferation and microcalli formation, the first bottleneck in protoplast regeneration. The use of these compounds represents an innovative strategy with high potential as new chemical enhancers of protoplast regeneration.

The molecules induced the up-regulation of the *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1-like* (*SERK1-like*) gene, and promoted somatic embryo formation in cork oak. *SERK1* is a well-known marker for the initiation of somatic embryogenesis whose expression increases during embryogenesis. *SERK1* is involved in the regulation of embryogenic competence (Hu et al., 2005). Arabidopsis seedlings overexpressing *AtSERK1* showed increased efficiency in initiating somatic embryogenesis (Hecht et al., 2001). Recent results from our group

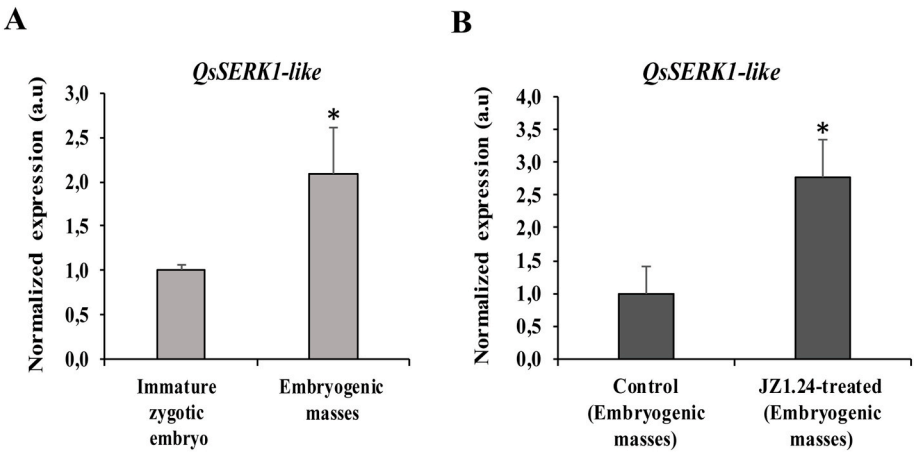

Fig. 6. Effect of LRRK2 inhibitor JZ1.24 on gene expression of *QsSERK1-like* in profiles during somatic embryogenesis induction of *Q. suber*. (A) Expression in control cultures, in immature zygotic embryos (initial explant, before somatic embryogenesis induction) and embryogenic masses (after induction). (B) Expression in embryogenic masses from control (untreated) cultures and in JZ1.24-treated embryogenic masses. Bars indicate the standard error of the mean, and asterisks indicate significant differences ($P \leq 0.05$) after Student's-*t*-test.

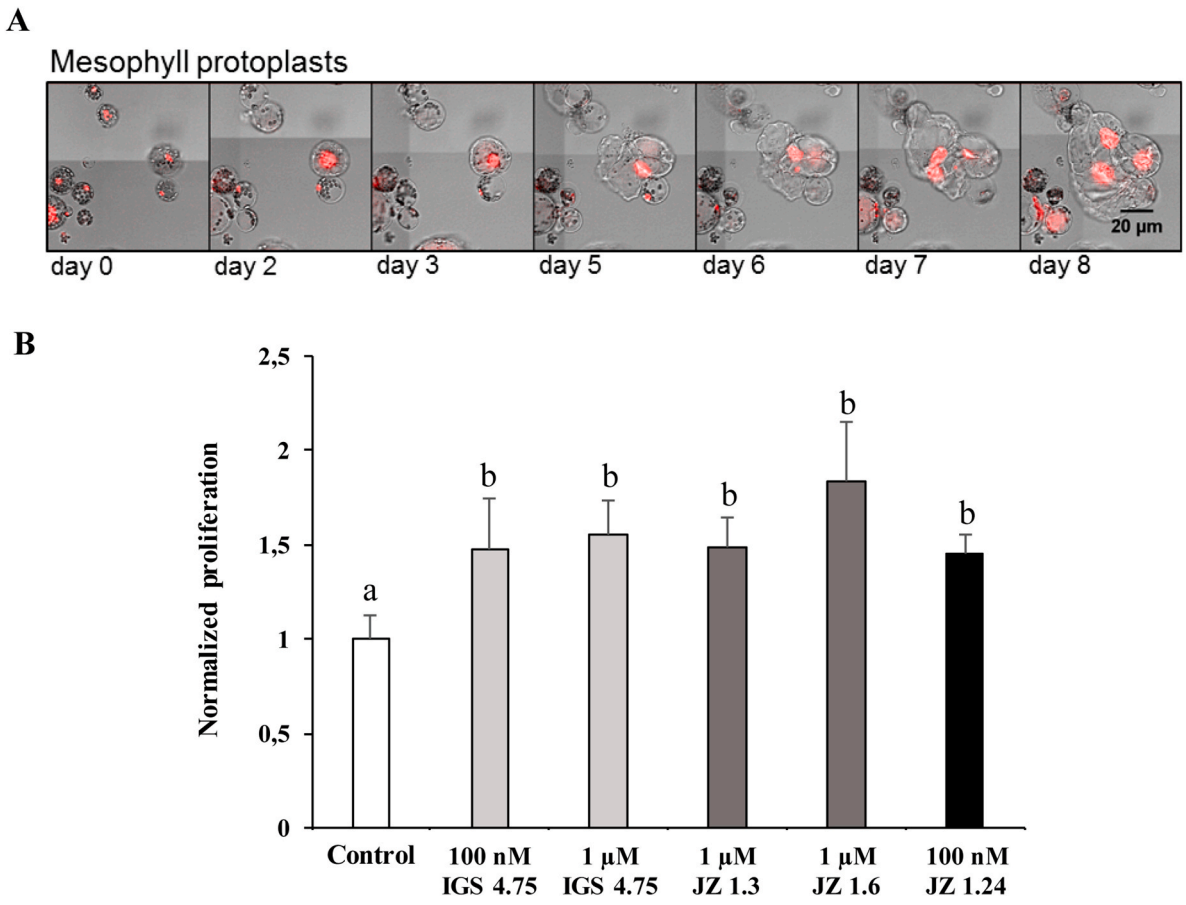

Fig. 7. Effect of LRRK2 inhibitors over proliferation of Arabidopsis protoplasts. (A) Tracking of proliferating protoplasts from mesophyll tissue of Arabidopsis seedlings expressing *H2B-YFP*. An exemplary image series of protoplasts isolated from mesophyll in cultivation medium is shown as overlay images from bright field and epifluorescence. The nuclear localised YFP fluorescence (red) indicates the position and number of nuclei and was used to identify proliferation events. (B) Quantification of proliferation events by image analysis, normalised to the total cell number cultivated and expressed relative to the proliferation rate of control cells treated with equivalent volume of DMSO. Data are means ± SEM from three biological replicates each. Different letters indicate significant differences according to ANOVA and Tukey's test at $P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

showed that the induction of somatic embryogenesis in cork oak correlates with the up-regulation of *QsSERK1- like* (Carneros et al., 2024). Furthermore, treatments with epigenetic modulators, which have been shown to promote the induction of somatic embryogenesis induction, also induces the expression of *QsSERK1- like* gene (Carneros et al., 2024).

Evidence underscores the critical role of hormonal regulation, including brassinosteroids, in plant somatic embryogenesis (Gou et al., 2012; Pérez-Pérez et al., 2019, 2023; Carneros et al., 2023). Previous reports have demonstrated that the exogenous application of brassinolide (the most active BR) enhances somatic embryogenesis progression in various species (Pullman et al., 2003; Ferrie et al., 2005; Belmonte et al., 2010; Chone et al., 2018). On the other hand, it has been shown that inhibiting BR biosynthesis with brassinazole in microspore cultures significantly impedes embryo formation, supporting the idea that endogenous BR is crucial during *in vitro* embryogenesis (Berenguer et al., 2021). Our findings indicate that LRRK2 inhibitors modulate the expression of BR-related genes. The changes in the expression profiles induced by the inhibitors align with an activation of the BR pathway (Nolan et al., 2020; Kour et al., 2021). Specifically, the application of the compounds resulted in decreased GSK3-BIN2 expression, thereby exogenously enhancing BR signaling. Results indicated that LRRK2 inhibitors reduced the expression of the feedback-regulated BR biosynthetic gene CPD, and increased the transcription of the BR-inducible gene BAS1, thus mimicking the feedback effects of BR on these pathway components (Nolan et al., 2020).

There is no plant orthologue of LRRK2. In human neural cells, LRRK2 has been reported to directly associate with the glycogen synthase kinase 3β, GSK-3β, and this interaction enhances the kinase activity of GSK-3β, suggesting that LRRK2 may act as an enhancer for GSK-3β (Kawakami et al., 2013; Kofoed et al., 2020). These reports suggested that small molecules inhibiting LRRK2 could have a negative impact on GSK3. In plants, the GSK3-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) is a negative regulator of the brassinosteroid (BR) signalling pathway (Nolan et al., 2020). A recent study showed that chemical inhibition of plant GSK3 activity by small molecule inhibitors of mammal GSK-3β lead to activation of the BR signalling pathway, increasing *in vitro* cell proliferation and somatic embryogenesis, in *B. napus* microspore cultures (Berenguer et al., 2021). In the absence of data identifying the plant target of the new compounds used in this study, we hypothesize that these compounds may inhibit an unidentified LRR-kinase activity that interacts with GSK3, thereby enhancing GSK3 activity, as observed in mammals. Consequently, LRRK2 inhibitors would negatively impact GSK3 activity of plant cells.

These findings provide new evidence for the potential of using small molecules from the human pharmaceutical field in plant cell reprogramming and regeneration systems. The results suggest that chemical modulation of kinase activities by small molecules can be used to overcome poor embryogenesis induction and cell proliferation in plant tissue culture. Further work is needed to understand the molecular targets and mechanism of action of these new compounds, and to investigate the extent these chemicals can help to improve *in vitro*

regeneration in other important species, a major bottleneck in modern plant breeding, opening up the possibility of transferring this knowledge to production, breeding and biotechnology programs.

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CRediT authorship contribution statement

Elena Carneros: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Eduardo Berenguer:** Methodology, Investigation, Formal analysis, Data curation. **Yolanda Pérez-Pérez:** Methodology, Investigation, Formal analysis, Data curation. **Saurabh Pandey:** Methodology, Investigation, Formal analysis, Data curation. **Ralf Welsch:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Klaus Palme:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Carmen Gil:** Writing – review & editing, Investigation, Formal analysis. **Ana Martínez:** Writing – review & editing, Investigation, Funding acquisition, Formal analysis. **Pilar S. Testillano:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2024.154334.

References

Belmonte, M., Elhiti, M., Waldner, B., Stasolla, C., 2010. Depletion of cellular brassinolide decreases embryo production and disrupts the architecture of the apical meristems in *Brassica napus* microspore-derived embryos. J. Exp. Bot. 61, 2779–2794. https://doi.org/10.1093/jxb/erq110.

Berenguer, E., Bárány, I., Solís, M.T., Pérez-Pérez, Y., Risueño, M.C., Testillano, P.S., 2017. Inhibition of histone H3K9 methylation by BIX-01294 promotes stress-induced microspore totipotency and enhances embryogenesis initiation. Front. Plant Sci. 8, 1161. https://doi.org/10.3389/fpls.2017.01161.

Berenguer, E., Carneros, E., Pérez-Pérez, Y., Gil, C., Martínez, A., Testillano, P.S., 2021. Small molecule inhibitors of mammalian GSK-3β promote *in vitro* plant cell reprogramming and somatic embryogenesis in crop and forest species. J. Exp. Bot. 72 (22), 7808–7825. https://doi.org/10.1093/jxb/erab365.

Berwick, D.C., Harvey, K., 2013. LRRK2: an eminence grise of Wnt-mediated neurogenesis? Front. Cell. Neurosci. 7, 82. https://doi.org/10.3389/fncel.2013.00082.

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J., Berger, F., 2001. Dynamic analyses of the expression of the HISTONE::YFP fusion protein in Arabidopsis show that syncytial endosperm is divided in mitotic domains. Plant Cell 13, 495–509. https://doi.org/10.1105/tpc.13.3.495.

Brocher, J., 2014. Qualitative and quantitative evaluation of two new histogram limiting binarization algorithms. Int. J. Image Process. 8, 30–48. https://doi.org/10.1016/j.clinph.2021.05.030.

Carneros, E., Sánchez-Muñoz, J., Pérez-Pérez, Y., Pintos, B., Gómez-Garay, A., Testillano, P.S., 2023. Dynamics of endogenous auxin and its role in somatic embryogenesis induction and progression in cork oak. Plants 12 (7), 1542. https://doi.org/10.3390/plants12071542.

Carneros, E., Díaz-Luzza, E.M., Pérez-Pérez, Y., Solís, M.T., Testillano, P.S., 2024. DNA methylation by transitory 5-azacytidine treatment improves somatic embryogenesis yield for regeneration and breeding in cork oak. Physiol. Plantarum 176, e14143. https://doi.org/10.1111/ppl.14143.

Che, P., Wu, E., Simon, M., Anand, A., Lowe, K., Gao, H., Sigmund, A., Yang, M., Albertsen, M.C., Gordon-Kamm, W., Jones, T.J., 2022. Wuschel2 enables highly efficient CRISPR/Cas-targeted genome editing during rapid de novo shoot regeneration in sorghum. Commun. Biol. 5 (1), 344. https://doi.org/10.1038/s42003-022-03308-w.

Chone, R.M.S., Rocha, D.I., Monte-Bello, C.C., Pinheiro, H.P., Dornelas, M.C., Haddad, C. R.B., Almeida, J.A.S., 2018. Brassinosteroid increases the cytokinin efficiency to induce direct somatic embryogenesis in leaf explants of *Coffea arabica* L. (*Rubiaceae*). Plant Cell Tiss. Organ Cult. 135, 63–71. https://doi.org/10.1007/s11240-018-1443-4.

Dovzhenko, A., Dal Bosco, C., Meurer, J., Koop, H.U., 2003. Efficient regeneration from cotyledon protoplasts in *Arabidopsis thaliana*. Protoplasma 222, 107–111. https://doi.org/10.1007/s00709-003-0011-9.

Falk, T., Mai, D., Bensch, R., Çiçek, Ö., Abdulkadir, A., Marrakchi, Y., Böhm, A., Deubner, J., Jäckel, Z., Seiwald, K., et al., 2018. U-Net: deep learning for cell counting, detection, and morphometry. Nat. Methods 16, 67–70. https://doi.org/10.1038/s41592-018-0261-2.

Feher, A., 2015. Somatic embryogenesis—stress-induced remodeling of plant cell fate. Biochim. Biophys. Acta 1849 (4), 385–402. https://doi.org/10.1016/j.bbagrm.2014.07.005.

Ferrie, A.M.R., Dirpaul, J., Krishna, P., Krochko, J., Keller, W.A., 2005. Effects of brassinosteroids on microspore embryogenesis in Brassica species. InVitro Cellular & Developmental Biol.-Plant 41, 742–745. https://doi.org/10.1079/IVP2005690.

In vitro embryogenesis in higher plants. In: Germanà, M.A., Lambardi, M. (Eds.), 2016. Methods in Molecular Biology. Springer, Cham, Switzerland. https://doi.org/10.1007/978-1-4939-3061-6.

Ginex, T., Madruga, E., Martinez, A., Gil, C., 2023. MBC and ECBL libraries: outstanding tools for drug discovery. Front. Pharmacol. 14, 1244317 https://doi.org/10.3389/fphar.2023.1244317.

Gordon-Kamm, B., Sardesai, N., Arling, M., Lowe, K., Hoerster, G., Betts, S., Jones, A.T., 2019. Using morphogenic genes to improve recovery and regeneration of transgenic plants. Plants 8 (2), 38. https://doi.org/10.3390/plants8020038.

Gou, X., Yin, H., He, K., Du, J., Yi, J., Xu, S., Lin, H., Clouse, S.D., Li, J., 2012. Genetic evidence for an indispensable role of somatic embryogenesis receptor kinases in brassinosteroid signaling. PLoS Genet. 8 (1), e1002452 https://doi.org/10.1371/journal.pgen.1002452.

Hecht, V., Vielle-Calzada, J.P., Hartog, M.V., Schmidt, E.D.L., Boutilier, K., Grossniklaus, U., de Vries, S.C., 2001. The Arabidopsis *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol. 127, 803–816. https://doi.org/10.1104/pp.010324.

Hu, H., Xiong, L., Yang, Y., 2005. Rice *SERK1* gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection. Planta 222, 107–117. https://doi.org/10.1007/s00425-005-1534-4.

Ibáñez, S., Carneros, E., Testillano, P.S., Pérez-Pérez, J.M., 2020. Advances in plant regeneration: shake, rattle and roll. Plants 9, 897. https://doi.org/10.3390/plants9070897.

Step wise protocols for somatic embryogenesis of important woody plants. In: Jain, S.M., Gupta, P. (Eds.), 2018. Forestry Sciences, 85. Springer, Cham, Switzerland. https://doi.org/10.1007/978-3-319-79087-9-27.

Kao, K.N., Michayluk, M.R., 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126, 105–110. https://doi.org/10.1007/BF00380613.

Kawakami, F., Shimada, N., Ohta, E., Kagiya, G., Kawashima, R., Maekawa, T., Maruyama, H., Ichikawa, T., 2013. Leucine-rich repeat kinase 2 regulates tau phosphorylation through direct activation of glycogen synthase kinase-3β. FEBS J. 281 (1), 3–13. https://doi.org/10.1111/febs.12579.

Kofoed, R.H., Betzer, C., Ferreira, N., Jensen, P.H., 2020. Glycogen synthase kinase 3 β activity is essential for Polo-like kinase 2- and Leucine-rich repeat kinase 2-mediated regulation of α-synuclein. Neurobiol. Dis. 136, 104720 https://doi.org/10.1016/j.nbd.2019.104720.

Kour, J., Kohli, S.K., Khanna, K., Bakshi, P., Sharma, P., Singh, A.D., Ibrahim, M., Devi, K., Sharma, N., Ohri, P., Skalicky, M., Brestic, M., Bhardwaj, R., Landi, M., Sharma, A., 2021. Brassinosteroid signaling, crosstalk, and physiological functions in plants under heavy metal stress. Front. Plant Sci. 12, 608061 https://doi.org/10.3389/fpls.2021.608061.

Kumlehn, J., Serazetdinova, L., Hensel, G., Becker, D., y Loerz, H., 2006. Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with Agrobacterium tumefaciens. Plant Biotechnol. J. 4 (2), 251–261. https://doi.org/10.1111/j.1467-7652.2005.00178.x.

Lichter, R., 1982. Induction of haploid plants from isolated pollen of Brassica napus. Z. Pflanzenphysiol. 105 (5), 427–434. https://doi.org/10.1016/S0044-328X(82)80040-8.

Loyola-Vargas, V.M., Ochoa-Alejo, N. (Eds.), 2018. Plant Cell Culture Protocols. Humana Press, New York. https://doi.org/10.1007/978-1-4939-8594-4_8.

Ma, X., Kong, L., Zhu, S., 2017. Reprogramming cell fates by small molecules. Protein Cell 8, 328–348. https://doi.org/10.1007/s13238-016-0362-6.

Maluszynski, M., Kasha, K.J., Forster, B.P., Szarejko, I. (Eds.), 2003. Doubled Haploid Production in Crop Plants. Springer, Dordrecht. https://doi.org/10.1007/978-94-017-1293-4_47.

Marum, L., Miguel, A., Ricardo, C.P., Miguel, C., 2012. Reference gene selection for quantitative real-time PCR normalization in *Quercus suber*. PLoS One 7, 9. https://doi.org/10.1371/journal.pone.0035113.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum 15 (3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Neumann, K.H., Kumar, A., Imani, J. (Eds.), 2009. Plant Cell and Tissue Culture- A Tool in Biotechnology. Principles and Practice. Springer, Berlin. https://doi.org/10.1007/978-3-540-93883-5_3.

Nolan, T.M., Vukašinović, N., Liu, D., Russinova, E., Yin, Y., 2020. Brassinosteroids: multidimensional regulators of plant growth, development, and stress responses. Plant Cell 32, 295–318. https://doi.org/10.1105/tpc.19.00335.

Olariu, V., Nilsson, J., Jönsson, H., Peterson, C., 2017. Different reprogramming propensities in plants and mammals: are small variations in the core network wirings responsible? PLoS One 12, e0175251. https://doi.org/10.1371/journal.pone.0175251.

Pérez-Pérez, Y., El-Tantawy, A.A., Solís, M.T., Risueño, M.C., Testillano, P.S., 2019. Stress-induced microspore embryogenesis requires endogenous auxin synthesis and polar transport in barley. Front. Plant Sci. 10, 1200. https://doi.org/10.3389/fpls.2019.01200.

Pérez-Pérez, Y., Solís, M.T., Albacete, A., Testillano, P.S., 2023. Opposite auxin dynamics determine the gametophytic and embryogenic fates of the microspore. Int. J. Mol. Sci. 24 (13), 11177 https://doi.org/10.3390/ijms241311177.

Preibisch, S., Saalfeld, S., Tomancak, P., 2009. Globally optimal stitching of tiled 3D microscopic image acquisitions. Bioinformatics 25, 1463–1465. https://doi.org/10.1093/bioinformatics/btp184.

Prem, D., Solís, M.T., Bárány, I., Rodríguez-Sanz, H., Risueño, M.C., Testillano, P.S., 2012. A new microspore embryogenesis system under low temperature which mimics zygotic embryogenesis initials, expresses auxin and efficiently regenerates doubled-haploid plants in *Brassica napus*. BMC Plant Biol. 12, 127. https://doi.org/10.1186/1471-2229-12-127.

Pullman, G.S., Zhang, Y., Phan, B.H., 2003. Brassinolide improves embryogenic tissue initiation in conifers and rice. Plant Cell Rep. 22, 96–104. https://doi.org/10.1007/s00299-003-0674-x.

Reed, K.M., Bargmann, O.R., 2021. Protoplast regeneration and its use in new plant breeding technologies. Front. Genome Ed 3, 734951. https://doi.org/10.3389/fgeed.2021.734951.

Rodríguez-Serrano, M., Bárány, I., Prem, D., Coronado, M.J., Risueño, M.C., Testillano, P. S., 2012. NO, ROS, and cell death associated with caspase-like activity increase in stress-induced microspore embryogenesis of barley. J. Exp. Bot. 63, 2007–2024. https://doi.org/10.1093/jxb/err400.

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinf. 18, 529. https://doi.org/10.1186/s12859-017-1934-z.

Salado, I.G., Zaldivar-Diez, J., Sebastian, V., Li, L., Geiger, L., González, S., Campillo, N. E., Gil, C., Morales, A.V., Perez, D.I., Martinez, A., 2017. Leucine Rich Repeat Kinase 2 (LRRK2) inhibitors based on indolinone scaffold: potential pro-neurogenic agents. Eur. J. Med. Chem. 138, 328–342. https://doi.org/10.1016/j.ejmech.2017.06.060.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

Schmidt, E.D., Guzzo, F., Toonen, M., de Vries, S.C., 1997. A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124, 2049–2062. https://doi.org/10.1242/dev.124.10.2049.

Teo, J.L., Kahn, M., 2010. The Wnt signalling pathway in cellular proliferation and differentiation: a tale of two coactivators. Adv. Drug Deliv. Rev. 62, 1149–1155. https://doi.org/10.1016/j.addr.2010.09.012.

Testillano, P.S., 2019. Microspore embryogenesis: targeting the determinant factors of stress induced cell reprogramming for crop improvement. J. Exp. Bot. 70 (11), 2965–2978. https://doi.org/10.1093/jxb/ery464.

Testillano, P.S., Gómez-Garay, A., Pintos, B., Risueño, M.C., 2018. Somatic embryogenesis from immature zygotic embryos in *Quercus suber* L. Methods Mol. Biol. 1815, 247–256. https://doi.org/10.1007/978-1-4939-8594-4_16.

Wittmer, J., Heidstra, R., 2024. Appreciating animal induced pluripotent stem cells to shape plant cell reprogramming strategies. J. Exp. Bot. 75, 4373–4393. https://doi.org/10.1093/jxb/erae264.

Yue, J.J., Yuan, J.L., Wu, F.H., Yuan, Y.H., Cheng, Q.W., Hsu, C.T., Lin, C.S., 2021. Protoplasts: from isolation to CRISPR/Cas genome editing application. Front. Genome Ed. 3, 717017 https://doi.org/10.3389/fgeed.2021.717017.

Zaldivar-Diez, J., Li, L., García, A.M., Zhao, W.N., Medina-Menendez, C., Haggarty, S.J., Gil, C., Morales, A.V., Martínez, A., 2020. Benzothiazole-based LRRK2 inhibitors as Wnt enhancers and promoters of oligodendrocytic fate. J. Med. Chem. 63, 2638–2655. https://doi.org/10.1021/acs.jmedchem.9b01752.