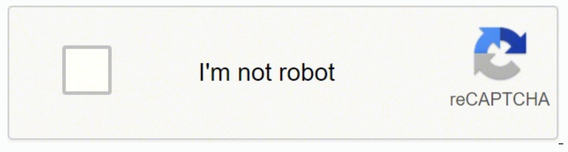

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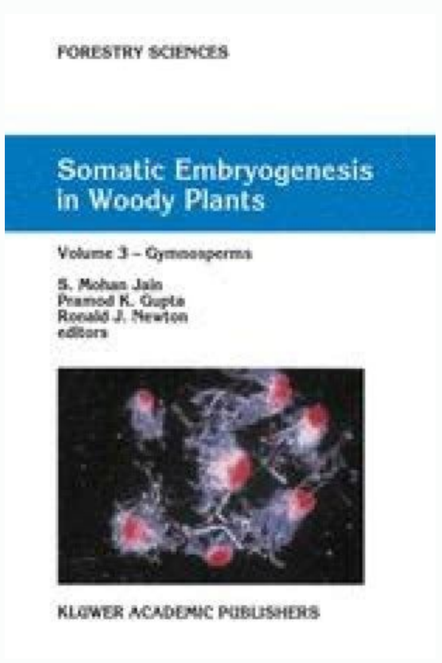
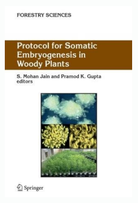
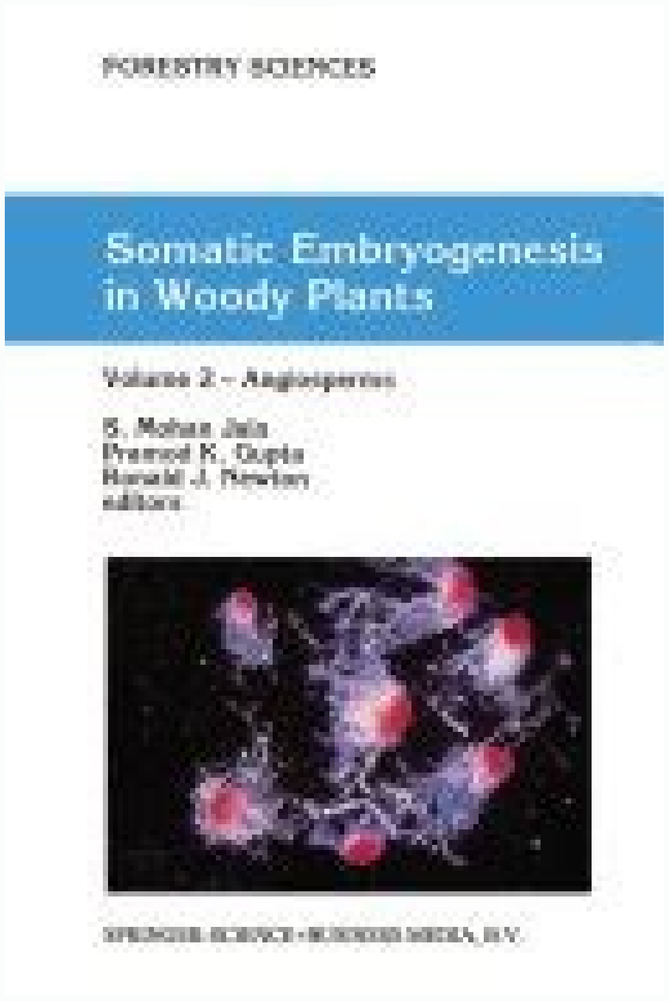
Somatic embryogenesis in woody plants pdf







20. Micropropagation of *Citrus*

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S.M. Jain and K.-Y. Hahn (eds.), Micropropagation of Woody Trees and Fruits, 337–419.

Induction of somatic embryogenesis in woody plants. Protocol for somatic embryogenesis in woody plants. Application of somatic embryogenesis in woody plants.

2006, 25, 183–189. Physiol. Isolation and characterization of a novel wheat cysteine-rich receptor-like kinase gene induced by Rhizoctonia cerealis. Efficient Transformation of Somatic Embryos and Regeneration of Cork Oak Plantlets with a Gene (CsTL1) Encoding a Chestnut Thaumatin-Like Protein. The expression of eight of them was quantified by digital PCR in Castanea genotypes showing different susceptibility to the pathogen after phenotyping through root inoculation with P. Holm oak (Quercus ilex L.) is the most characteristic tree species in Mediterranean forests, with the largest areas of cover located in the Iberian Peninsula [5]. Solla (Univ. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (. 2009, 4, 1010–1012. For each WT and transgenic line, 36 somatic embryos were cultured per jar (6 embryos per jar). This medium consisted of GD mineral medium [65] supplemented with 0.1 mg/L 6-benziladenine (BA) and 20 µM silver thiosulfate. Next, 1 mL of this bacterial suspension was used to inoculate 600 mL of liquid LB medium with 50 mg/L kan. The oomycete Phytophthora cinnamomi Rands is considered to be the main cause of oak decline, but the influence of other biotic and abiotic factors related to climate change accelerates the symptoms [7]. PLoS ONE 2021, 7, e35113. The role of climate change in the widespread mortality of holm oak in open woodlands of Southwestern Spain. A BLASTn was performed to the primer pair against known Quercus genomes in the National Center for Biotechnology Information (NCBI), with no predicted alignment to specific genes. Online resource 3. cinnamomi, the main cause of oak decline. [Google Scholar] [CrossRef]Sambrook, J.; Fritsch, E.F.; Maniatis, T. Plant Pathol. However, the present study reports the successful transformation of holm oak somatic embryos with the Cast_Gnk2-like gene. New For. Pollut. From the outermost area from the plate, where growth was most active, discs of 5 mm agar with the help of a punch and placed on a Petri dish (9 cm diameter) with 25 mL of clarified V8 medium. During that time, GFP expression was also checked periodically to rule out the appearance of chimeras. qPCR conditions were the same used in [45]: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 58 °C for 1 min. Online resource 2. Despite the estimation of two copies of the transgene in Q8-Gin2, double of what was found in the other transgenic lines, it was not reflected in a significantly higher expression of the Cast_Gnk2-like gene. [54] recently reported that the overexpression of plant cysteine-rich receptor-like kinase in pepper and tobacco produces an antifungal effect against Ralstonia solanacearum, similar to that caused by ginkbilobin in Ginkgo biloba. This indirect approach, i.e., the transformation with unspecific genes, was applied as it was not known which genes specifically control tolerance to the most important pathogens of these tree species. 1993, 2, 208–218. [Google Scholar] [CrossRef]Guan, Y.; Li, S.-G.; Fan, X.-F.; Su, Z.-H. High-efficiency Agrobacterium-mediated transformation in Quercus robur: Selection by use of a temporary immersion system and assessment by quantitative PCR. Propagation of mature Quercus ilex L. After incubation for 24 h in darkness at 24 °C, the medium V8 was replaced by 25 mL of Chen and Zentmyer salt solution [69]. 2008, 27, 865–872. As in other transformation systems [19,39,40], transformation efficiency in holm oak is affected by the genotype of the embryogenic lines, and transformation was only obtained in two of the four lines evaluated. cinnamomi. To confirm the correct orientation of the Cast_Gnk2-like cDNA in the pENTRTM/D-TOPO® plasmid, PCR amplification was carried out and the entire insert was subsequently sequenced. [Google Scholar] [CrossRef]Martínez, M.T.; San-José, M.C.; Arrillaga, I.; Cano, V.; Morcillo, M.; Cernadas, M.J.; Corredoira, E. These results seem to be correlated with the Cast_Gnk2-like expression data since the Q8-Gin1 and E2-Gin2 lines showed the highest levels of expression (Figure 3). The plants were examined daily and a plant was considered dead when all organs were 100% necrotic. Forests 2011, 2, 486–504. crenata are taxonomically closely related, the nucleotide sequences of the Gnk2-like gene must have high similarity. Once the transgenic lines were established, they were also cryopreserved according to [18] for indefinite conservation while their transformation and tolerance were tested.Once the transgenic lines were established, different molecular techniques were applied to confirm the presence of Cast_Gnk2-like gene, the gene copy number and the transcription and expression of the gene in the transgenic lines of holm oak.The presence of the transgenes NPTII and GFP was confirmed in all transgenic lines analyzed and in the plasmid (positive control) but not in the non-transgenic lines (WT) (negative control) (Figure 2a,b). Cucumber transgenic plants overproducing the Gnk2-1 gene have displayed resistance to Fusarium oxysporum [34]. [Google Scholar] [CrossRef]Stotz, H.U.; Thomson, J.; Wang, Y. crenata root surrounding may be a hostile environment for fungal and fungal-like pathogens, such as P. Cuad. (a) Appearance of kan-resistant embryos developed on selection medium 10 weeks after transformation. 1998, 1, 142–148. Despite their different origin, the 4 embryogenic lines show a similar behavior during proliferation and germination steps. Fluorescence was observed using a Leica M205 FA (Leica, Wetzlar, Germany) stereomicroscope, equipped with a 200-W bulb, and a specific filter for fluorescence, with 470/40× nm of excitation and 525/50 nm of emission. crenata was cloned into the plasmid pK7WG2D under the CaMV35S promoter utilizing the Gateway recombination cloning technology (Thermo Fisher Scientific, Waltham, MA, USA). [Google Scholar] [CrossRef]Cano, V.; Martínez, M.T.; San José, M.C.; Couselo, J.L.; Varas, E.; Bouza-Morcillo, L.; Toribio, M.; Corredoira, E. J. Table 3. Environ. Licensee MDPI, Basel, Switzerland. crenata Gnk-2 like protein was achieved in Q. Similarly, line E2-Gin1 survived significantly more days (8.1 days) than the E2-WT line (6.69 days) (Figure 4). [Google Scholar] [CrossRef] [PubMed]Mallón, R.; Vieitez, A.M.; Vidal, N. Overexpression of the Chestnut CsTL1 Gene Coding for a Thaumatin-like Protein in Somatic Embryos of Quercus robur. In addition, in vitro tests ensure that all plants are of the same age and are physiologically homogeneous. [Google Scholar] [CrossRef]Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT–PCR. 2020, 11, 308. [Google Scholar] [CrossRef]Corcobado, T.; Cubera, E.; Moreno, G.; Solla, A. [Google Scholar] [CrossRef]Sicard, P.; Augustaitis, A.; Belyazid, S.; Calfapietra, C.; de Marco, A.; Fenn, M.; Bytnerowicz, A.; Grulke, N.; He, S.; Matyssek, R.; et al. The authors also thank A. Biochem. cinnamomi.Ginkbilobin-2 (Gnk2) is a seed storage protein present in gymnosperm Ginkgo biloba seeds that possesses an antifungal activity. In the transformed lines, the correlation between the CT and the quantity of plasmid that mimicked different copy numbers of the CaMV35S promoter pointed to one copy in lines Q8-Gin1 and E2-Gin1, two copies in Q8-Gin2 and, as expected, no copies in non-transformed genotypes (wild-type WT, Table 2). [Google Scholar] [CrossRef]Mallón, R.; Valladares, S.; Corredoira, E.; Vieitez, A.M.; Vidal, N. Kan-resistant explants were isolated and transferred to selection medium but supplemented with 125 mg/L kan rather than 100 mg/L initially. [Google Scholar] [CrossRef]Corredoira, E.; San-José, M.C.; Vieitez, A.M.; Ballester, A. Subsequently, explants were immersed in infection medium during 30 min with gentle shaking. [Google Scholar] [CrossRef]Cuenca, B.; Ocaña, L.; Salinero, M.C.; Pintos, C.; Mansilla, J.P.; Rial, C. [Google Scholar] [CrossRef]Leroy, T.; Henry, A.M.; Royer, M.; Altosaar, I.; Frutos, R.; Duris, D.; Philippe, R. In an in vitro tolerance assay with the pathogen P. Heterologous Expression of the AtNPR1 Gene in Olive and Its Effects on Fungal Tolerance. Lisboa) for the use of the CFX96 qPCR equipment.The authors declare no conflict of interest.Stenlid, J.; Oliva, J.; Boberg, J.B.; Hopkins, A.J.M. Emerging Diseases in European Forest Ecosystems and Responses in Society. The in vitro behaviour and growth of these transgenic plants were not affected by overexpression of the Cast_Gnk2-like gene. Acta Hortic. 2020, 51, 1003–1021. Rep. It binds with high affinity to D-mannose and with less affinity to D-glucose, and both exist in the hyphal cell walls of Phytophthora species [25]. Can. After infection, transgenic plants survived more days than non-transformed plants. Likewise, for avocado, another species considered recalcitrant to transformation, a transformation rate of only 10% has been reported [43].The selection efficiency was relatively high in the present study, especially in line E2. Bars are mean ± standard error (n = 3). After eight weeks of culture under standard conditions, the following parameters were determined: percentage of embryos that only developed a root ≥ 5 mm and the percentage of embryos that develop a complete plant, as well as the root (mm) and shoot (mm) lengths and the number of leaves per plant. ilex, there are no previous reports that reveal a reference gene or a transgenic line for which the copy number had been estimated. The correct product was transferred into the plasmid pK7WG2D employing the Gateway™ LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific, Waltham, MA, USA) as recommended the manufacturer. Cast_Gnk2-like revealed to be the most expressed gene across all experiments and the one that best discriminates between susceptible and resistant genotypes [23]. [35] were recently successful in overexpressing the Cast_Gnk2-like gene in American chestnut somatic embryos, but the tolerance of transgenic plants to P. Development and characterization of EST-SSR markers for mapping reaction to Phytophthora cinnamomi in Castanea spp. Global topics and novel approaches in the study of air pollution, climate change and forest ecosystems. 57/2016, as well as through research funds awarded to RM (PTDC/BIA-FBT/28170/2017) and to BioISI (UIDB/04046/2020 and UIDP/04046/2020).The authors thank M. Then, the bacterial culture was centrifuged at 6500 rpm for 10 min at 10 °C, and the pellet obtained was resuspended in 200 mL of liquid MS mineral medium supplemented with 5 g/L sucrose (infection medium).For the genetic transformation, 2 or 3 individualized PEMs isolated from 6-week-old cultures after the last subculture were pre-cultured for 1 week on proliferation medium. Therefore, the CaMV35S promoter of viral origin that drives the expression of Cast_Gnk2-like gene in transgenic lines was selected to compare the copy number of the inserted gene. The results validate GFP fluorescence as an effective, precise and non-destructive marker for detecting transgenic somatic embryos in holm oak. Biophys. Eukaryot. The green fluorescent protein as an efficient selection marker for Agrobacterium tumefaciens-mediated transformation in Hevea brasiliensis (Müll. Melt curve analysis in qPCR reactions for copy number estimation. Southern blotting constitutes the most precise technique to evaluate copy number, but it requires high amounts of genomic DNA, skills and is time-consuming. For confirming the presence of the Cast_Gnk2-like gene, two fragments of different lengths were amplified, the shorter (890 bp) including part of the T-35S terminator and part of the Cast_Gnk2-like sequence, whereas the larger (1227 bp) included part of the CaMV35S promoter and the sequence of the Cast_Gnk2-like gene (see Online resources 1 and 2). 2010, 29, 1251–1260. [Google Scholar] [CrossRef]Palomo-Ríos, E.; Cerezo, S.; Mercado, J.A.; Pliego-Alfaro, F. Sci. 2007, 8, 105–112. After the incubation period, the correct sporangia formation was verified with stereomicroscope and to induce synchrony sporulation and the simultaneous release of zoospores in the tube Petri dishes were incubated for 15–30 min at 4 °C just before starting the plant infection experiment.To evaluate the tolerance of transgenic holm oak plants to P. Forest 2021, 12, 1634. Student’s t-test was used for statistical analysis.Maturation and germination of somatic embryos from non-transgenic and transgenic lines was performed according the procedure described by Martínez et al. [Google Scholar]Chen, S.C.; Cannon, C.H.; Kua, C.S.; Liu, J.-J.; Galbraith, D.W. Genome size variation in the Fagaceae and its implications for trees. cinnamomi, we observed that transgenic plants were able to survive longer than wild type. Oak decline consists of the death of thousands of cork oaks and holm oaks, which are keystone species in the dehesas. [Google Scholar] [CrossRef] [PubMed]Andrade, G.M.; Nairn, C.J.; Le, H.T.; Merkle, S.A. Sexually mature transgenic American chestnut trees via embryogenic suspension-based transformation. A Secreted Protein with Plant-Specific Cysteine-Rich Motif Functions as a Mannose-Binding Lectin That Exhibits Antifungal Activity. A similar problem has also been mentioned in regard to gene expression in other woody species such as European chestnut [16], olive [48] and cork oak [19]. Figure 3. The selected embryos were proliferated in selection medium. [Google Scholar] [CrossRef]Kotrade, P.; Sehr, E.M.; Wischnitzki, E.; Brüggemann, W. [Google Scholar] [CrossRef]Stevens, R.B. Mycology Guidebook; University of Washington Press: Seattle, WA, USA, 1974; 703p. Plant 2018, 54, 341–376. Such programmes will probably never be carried out as the species has a long reproductive cycle, and thus long periods are required to complete one cycle of conventional improvement [10]. 9.7 ng of cDNA were used per reaction in a 15 µL final volume using 7.5 µL of Maxima SYBR Green qPCR Master Mix kit (Thermo Fisher Scientific, Waltham, MA, USA). Additionally, for each embryogenic line, 20 not infected explants were cultured in proliferation medium without antibiotics (control positive) and with antibiotics (negative control).After 10 weeks of culture on selective medium, the rate of kan-resistant explants defined as the percentage of initial explants showing the formation of new somatic embryos and/or embryogenic structures was recorded. Appl. Bull. 2007, 91, 281–288. [Google Scholar] [CrossRef]Merkle, S.A.; Dean, J.F.D. Forest tree biotechnology. After germination, leaves and roots from transgenic plantlets and their non-transformed counterparts were also examined for stable GFP expression.The P. The presence of transgenes was determined by PCR screening using the primer pairs specific to (a) NPTII (472 bp), (b) GFP (740 bp), (c) Cast_Gnk2-like-R fragment (890 bp) and (d) Cast_Gnk2-like-F fragment (1227 bp). Total RNA (2 µg) was used as template for reverse transcription with RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and primed with an oligo(dT) primer. A final concentration of 0.2 µM of each primer was used in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Dev. Primers and amplification programs utilized in the present report. Cloning mature holm oak trees by somatic embryogenesis. ilex do not bear the viral CaMV35S promoter, the detected amplicon in WT points to an unspecific amplification.Cast_Gnk2-like gene expression in each transformed line was evaluated in somatic embryos at early cotyledonary stage by qPCR (Figure 3). Commun. Figure 5. Thereafter, in both experiments the statistical significance was tested using analysis of variance one way (ANOVA I) and multiple mean comparisons were performed by Tukey’s HSD (honestly significant difference) test. The medium was dispensed into Petri dishes (9 cm in diameter) and 10–12 individualized proembryogenic masses were cultured on each dish.Unless specified, the cultures were maintained in a photoperiodic climatic chamber with a 16 h light and 8 h dark photoperiod (standard conditions). The presence of no more than two copies of the transgene may avoid silencing events the in the transformed lines.The WT genotypes resulted in a detected CT, however, it was remarkably higher than the CT obtained for the one copy transformed lines. 2005, 12, 13–18. Among lines derived from Q8 lines, conversion rates ranged from 30.6% to 69.5% and without significant differences to one another (Table 3). ilex non-transformed genotypes was lower than the expression level obtained for the respective transformed lines. 2001, 29, e45. In Vitro Cell. The very low copy number of the T-DNA inserted in these two lines makes them more suitable for further functional analysis, as there is a low probability of transgene inactivation

[36,51].Moreover, the level of disease tolerance was positively correlated with the level of expression of the Cast_Gnk2-like gene. The images were taken with a Leica DSC7000T camera (Leica, Wetzlar, Germany). Transgene and its expression in transgenic plants: A review. [Google Scholar] [CrossRef]Flavell, R.B. Inactivation of gene expression in plants as a consequence of specific sequence duplication. [Google Scholar] [CrossRef]Natalini, F.; Alejano, R.; Vázquez-Piqué, J.; Cañellas, I.; Gea-Izquierdo, G. Dendrochronologia 2016, 38, 51–60. [21] in infected roots of C. For each line, a total of six plants were infected, and the experiment was repeated thrice (i.e., 18 plants per line).Statistical analyses were accomplished using the SPSS 26 software (SPSS Inc., Chicago, IL, USA). No GFP fluorescence was observed in non-transformed shoots and roots subjected to the same excitation and emission conditions than transgenic material. crenata in somatic embryos of holm oak increases the tolerance of the trees to P. Subsequently, 4 washes with the salt solution every 30 min were performed. After the last wash, the mycelium discs remained in the dish, bathed with the salt solution under black light at 24 °C for 24 h. [Google Scholar] [CrossRef]Wang, H.; Ng, T.B. Ginkbilobin, a novel antifungal protein from Ginkgo biloba seeds with sequence similarity to embryo-abundant protein. Both fragments were not amplified in non-transformed counterparts (Figure 2c,d).The Cast_Gnk2-like copy number in each transformed line was estimated through its promoter CaMV35S, by qPCR, in the lines formerly analyzed by PCR. 2015, 99, 4961–4981. Therefore, improving holm oak tolerance to the disease has emerged as a subject of great research interest, particularly with respect to transformation studies. However, great advances have been made in genomic tools in recent years, and it is now possible to identify candidate genes that are directly involved in resistance in these species [20]. Reactions started with a denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing temperature for 30 s. [Google Scholar] [CrossRef] [PubMed]Haavik, L.J.; Billings, S.A.; Guldin, J.M.; Stephen, F.M. Emergent insects, pathogens and drought shape changing patterns in oak decline in North America and Europe. Hist. Microbiol. Figure 5. [Google Scholar] [CrossRef] [PubMed]Chang, S.; Mahon, E.L.; MacKay, H.A.; Rottmann, W.H.; Strauss, S.H.; Pijut, P.M.; Powell, W.A.; Coffey, V.; Lu, H.; Mansfield, S.D.; et al. 2016, 213, 977–987. As generally described in most genetic transformation systems, the level of transgene expression varied greatly among the different transgenic lines generated. Plants transformed with Cast_Gnk2-like gene grew normally and were morphologically indistinguishable from non-transformed controls (Online resource 4).Transformed plants of holm oak and its untransformed counterparts obtained as described above were infected in order to determine if the overexpression of Cast_Gnk2-like gene could improve the tolerance against P. In the present study, we found that the overexpression of the Cast_Gnk2-like gene conferred a moderate level of tolerance to P. Based on the ratio of transgene copy number to the size of Q. Micropropagation, characterization and conservation of Phytophthora cinnamomi-tolerant holm oak mature trees. Each set of reactions included a no template control, two technical replicates and two repetitions. PEMs were co-cultured for 5 days with Agrobacterium EHA105pGnk2 and then cultured on selective medium containing kanamycin (kan) and carbenicillin. The culture was grown overnight at 28 °C with shaking (180–200 rpm) in darkness. This gene codifies a seed storage protein present in the gymnosperm of Ginkgo biloba seeds. Agrobacterium-mediated genetic transformation of Coffea arabica (L.) is greatly enhanced by using established embryogenic callus cultures. In a near future, experiments with ex vitro plants are needed to confirm the tolerance to P. [Google Scholar] [CrossRef]Santos, C.; Zhebentyayeva, T.; Serrazina, S.; Nelson, C.D.; Costa, R. Plant Physiol. Tree Physiol. However, and despite the obvious antifungal properties of ginkbilobin, this protein has scarcely been studied, and there are very few reports of genetic transformation with this protein. Drought events determine performance of Quercus ilex seedlings and increase their susceptibility to Phytophthora cinnamomi. On the contrary, the expression in Q8-Gin2 was the lowest amongst the transgenic lines.After cold storage and germination treatments, root-only development and whole plant recovery (Figure 1d) were obtained in somatic embryos of four holm oak transgenic lines and in their corresponding wild-type lines (Table 3). Planta 1972, 107, 161–170. Soc. 2013, 114, 171–185. Gene expression was calculated using the Pfaffl method [64]. [27], we established a standard curve by mixing the plasmid carrying the transgene used for transformation (pK7WG2D-Gnk2) with non-transformed genomic DNA. Analyses of Extracellular Carbohydrates in Oomycetes Unveil the Existence of Three Different Cell Wall Types. Genetically modified coffee plants expressing the Bacillus thuringiensis cry 1Ac gene for resistance to leaf miner. Non-transformed data are presented in the Tables and Figures. Miyakawa et al. Mol. CRK2 and C-terminal Phosphorylation of NADPH Oxidase RBOHD Regulate Reactive Oxygen Species Production in Arabidopsis. In the present study, we investigated whether overexpression of the Cast_Gnk2-like gene from C. Somatic embryogenesis is considered the best regeneration method for producing transgenic plants in hardwood species as the regeneration capacity is higher and the incidence of chimera is lower than in other regeneration methods [15]. 2012, 32, 1389–1402. This type of material has already been suggested to be the most suitable target tissue for the transformation of several woody species such as Vitis rotundifolia [38], C. Trees 2014, 28, 657–667. [Google Scholar] [CrossRef] [PubMed]Dutt, M.; Grosser, J.W. An embryogenic suspension cell culture system for D-mediated transformation of citrus. cinnamomi in a non-transformed plantlet (a,c) and transformed plantlet (b,d) of genotype Q8 immediately after inoculation (a,b) and 7 days later (c,d). Table 2. crenata were compared after P. 2008, 26, 171–176. Forests 2020, 11, 1196. The DNA of putatively transgenic lines produced both fragments after the amplification by PCR, confirming the transgenic nature of these lines (Figure 2c,d). Figure 2. 2014, 166, 766–778. Genetic engineering of trees: Progress and new horizons. 2002, 20, 948–954. crenata are taxonomically close, the endogenous Gnk2-like gene in Q. M DNA ladder; P corresponds to plasmid DNA (positive control); Lanes 1–2: E2 amplification corresponding non-transformed (1) and transformed (2) lines; Lanes 3–5: Q8 amplification corresponding non-transformed (3) and transformed (4, 5) lines. After that time, the infection medium was removed by the filtration and the explants were transferred to proliferation medium for 5 days of coculture in the dark at 25 °C. [Google Scholar] [CrossRef]Liu, J.; Tian, H.; Wang, Y.; Guo, A. Lines E2, Q8 and E00 were induced from teguments of ovules derived from adult trees [55], while the Q10-16 line was initiated in a leaf excised from axillary shoot cultures established from a centenary holm oak [50]. Low transgene copy numbers have been described in other embryogenic systems of several woody species [36,39,49]. (holm oak) trees by somatic embryogenesis. Agrobacterium-mediated transformation of avocado (Persea americana Mill.) somatic embryos with fluorescent marker genes and optimization of transgenic plant recovery. Overexpression of a Novel Antifungal Protein Gene GNK2-1 Results in Elevated Resistance of Transgenic Cucumber to Fusarium oxysporum. In each reaction, 2 ng of DNA were used in a 20 µL final volume using 10 µL of Sensi Fast SYBR Hi-ROX kit (Bioline, Meridian Bioscience, Cincinnati, OH, EUA) and 0.2 µM of each primer in a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Quercus ilex forests are influenced by annual variations in water table, soil water deficit and fine root loss caused by Phytophthora cinnamomi. Holm oak belongs to the group of woody species that are considered recalcitrant to regeneration and transformation [10]. This is particularly true in the case of woody species, many of which are recalcitrant to transformation [36] and references therein]. USA 1994, 91, 3490–3496. 2015, 354, 190–205. 2016, 5, e003. Seven of the eight candidate genes displayed differentially expressed levels depending on genotype and time-point after inoculation. For example, transformation efficiency of less than 1% was observed in the recalcitrant species C. Tree Genet. The aim of the present study was to produce holm oak plants that overexpress the Ginkbilobin-2 homologous domain gene (Cast_Gnk2-like) that it is known to possess antifungal properties. Embryogenic LineKanamycin Resistant Explants (%) 1Transformation Efficiency (%) 2Selection Efficiency (%) 3Q85.0 ± 2.72.5 ± 1.750.0E23.3 ± 1.42.5 ± 1.375.0Q10-160.0 ± 0.00.0 ± 0.0-E000.0 ± 0.00.0 ± 0.0-χ2p < 0.05ns- Table 2. One of the ecosystems thus affected is the Spanish dehesas and Portuguese montados (dehesas, hereafter), which are experiencing an unprecedented crisis, fundamentally caused by a complex disease commonly known as oak decline [3]. Nucleic Acids Res. The binary plasmid, called pK7WG2D-Gnk2 (Online resource 1), was transferred to Agrobacterium tumefaciens strain EHA105 [58] by the freeze-thaw method [59] and was used in the transformation procedures.The cultures of the EHA105pK7WG2-Gnk2 strain were started at from a glycerol stock stored at −80 °C and were grown overnight at 28 °C, with stirring (180–200 rpm), in a Luria–Bertani liquid (LB: 10 g/L tryptone, 0.5 g/L yeast extract and 10 g/L of NaCl, pH 7.0) [60] supplemented with 50 mg/L of kanamycin. Conventional breeding methods must be complemented with biotechnological tools to produce superior trees with enhanced disease tolerance within a shorter time. Genetic Transformation of European Chestnut Somatic Embryos with a Native Thaumatin-Like Protein (CsTL1) Gene Isolated from Castanea sativa Seeds. The highest Cast_Gnk2-like expression registered in devoid of oomycete conditions also suggests that C. [Google Scholar] [CrossRef]Santos, C.; Duarte, S.; Tedesco, S.; Fevereiro, P.; Costa, R.L. Expression Profiling of Castanea Genes during Resistant and Susceptible Interactions with the Oomycete Pathogen Phytophthora cinnamomi Reveal Possible Mechanisms of Immunity. Plant. Plant Cell Tissue Organ Cult. [Google Scholar] [CrossRef]Pelegrini, P.B.; Franco, O.L. Plant gamma-thionins: Novel insights on the mechanism of action of a multi-functional class of defense proteins. 1972, 50, 199–204. This syndrome has drastically affected the sustainability of the dehesa ecosystem, which is unique within Europe, and the situation is of great concern due to the associated high economic, ecological and social losses [4]. However, the expression level obtained for the two Q. In the present study, expression of a C. 2000, 19, 382–385. BMC Plant Biol. Bar: 1 mm. [50]. The strain was kept in V8-Agar medium [67] with replicates every 2–3 weeks. Transgenic plants were obtained from all transgenic lines after cold storage of the somatic embryos for 2 months and subsequent transfer to germination medium. cinnamomi A2 strain, named UEX-1, used in the tolerance experiments, was isolated from the infected roots of holm oak trees from Valverde de Mérida (Badajoz, Spain) [66]. Variaciones en la susceptibilidad a Phytophthora cinnamomi de diferentes clones de castaño: Comparación de dos métodos de inoculación. Online resource 4. Once the UEX-1 strain was reactivated, it was grown for one week in disposable Petri dishes (9 cm diameter) with 25 mL V8 medium without antibiotics in darkness at 24 °C. Planta 2006, 224, 1373. [Google Scholar] [CrossRef]Schenk, R.U.; Hildebrand, A.C. Medium and techniques for induction of growth of monocotyledonous and dicotyledonous plant cell culture. Behav. 1962, 15, 473–497. Plant cysteine-rich receptor-like kinases are known to be involved in the regulation of immune responses [52,53]. Application of Somatic Embryogenesis in Woody Plants. For example, to elucidate chestnut defense mechanisms to ink disease, root transcriptomes of the susceptible species Castanea sativa and the resistant species C. Low transformation rates (0.3–4%) were also obtained in a recalcitrant pear cultivar [42]. An in vitro test used to evaluate its overexpression can improve the tolerance of holm oak plants against the infection of P. 2010, 866, 111–119. Plant Cell Rep. Biotechnol. Protein Pept. Thus, the tolerance to P. Several antifungal proteins such as defensins, lipid tranfer proteins and thionins have been successfully overexpresed in order to confer tolerance to diseases caused by different fungi [31,32,33]. dentata [39] and Coffea arabica [36]. Digit. Molecular Cloning: A Laboratory Manual, 2nd ed.; Cold Spring Harbor Laboratory Press: Nueva York, NY, USA, 1989. [Google Scholar] [CrossRef]Dhekney, S.A.; Li, Z.T.; Dutt, M.; Gray, D.J. Agrobacterium-mediated transformation of embryogenic cultures and plant regeneration in Vitis rotundifolia Michx. ilex and C. Somatic embryos at cotyledonary stage (≥5 mm) were isolated from 6-week-old cultures and transferred to empty Petri dishes (9 cm in diameter), where they remained in semi-dark conditions for two months at 4 °C. Mycologia 1970, 62, 397–402. Of the three transgenic lines, only in the Q8-Gin3 line were the conversion percentages lower than those registered in the non-transformed line. The correlation efficiencies of the standard curve obtained as in [27] and described in the Material and Methods were between 0.993 and 0.996, and the efficiency of the reactions was between 94 and 97%. Each set of reactions included a no template control and three technical replicates. However, traditional improvement programmes involving selective crossing to produce trees that are resistant/tolerant to the disease have not been carried out. [Google Scholar] [CrossRef] [PubMed]Cox, K.D.; Layne, D.R.; Scorza, R.; Schnabel, G. In addition, not significant differences were found in the shoot length and leaf number.Leaves and roots of the regenerated plantlets were evaluated regarding GFP expression. Figure 2. The following supporting information can be downloaded at: Online resource 1. The most effective means appears to be the afforestation of affected zones with genotypes that are tolerant to P. In recent decades, global climate change has greatly exacerbated the effects of many plant diseases, especially fungal diseases, which can severely affect forest ecosystems [1,2]. (muscadine grape). 2021, 22, 1757. Screening of transgenic embryos by PCR. [Google Scholar] [CrossRef]FAO; UNEP. Morphological appearance and progress of infection with P. In total, four transgenic lines were obtained, three lines from the Q8 line and one line from the E2, which were maintained by secondary embryogenesis. ilex genome (1.98 pg/2C, [61]) we calculated the quantity of plasmid needed to be mixed with 2 ng of non-transgenic Q. Sporulation started approximately 8 h after the last wash, and reached its level maximum between 24 and 36 h from onset. Castanea root transcriptome in response to Phytophthora cinnamomi challenge. Arg). The advantages of in vitro tests include the fact that the rapid execution enables several repetitions to be made in a short period of time and only rooting of regenerated shoots is required. ilex may have a high level of similarity with Cast_Gnk2-like, and both genes can be amplified in the same reaction. Faculdade de Ciências, BioISI—Biosystems & Integrative Sciences Institute, Universidade de Lisboa, 1749-016 Lisbon, Portugal Misión Biológica de Galicia, Consejo Superior de Investigaciones Científicas (MBG-CSIC), Avda Vigo s/n, Campus Vida, Apartado 122, 15705 Santiago de Compostela, Spain Instituto Nacional de Investigação Agrária e Veterinária, Avenida da República, Quinta do Marquês, 2780-159 Oeiras, Portugal Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisbon, Portugal Author to whom correspondence should be addressed. Morphological appearance of non-transformed plantlets and transformed plantlets obtained after embryo germination.Conceptualization, E.C.; investigation, E.C., S.S., M.T.M. and V.C.; writing—original draft preparation, E.C., R.L.C. and S.S.; writing—review and editing, E.C., S.S., M.T.M., R.L.C. and R.M.; resources to S.S., R.M. All authors have read and agreed to the published version of the manuscript.This investigation was partly supported by the Spanish govern through the projects AGL2016-76143-C4-4-R and PID2020-112627RB-C33 (MICINN), and by the Portuguese govern through the projects PTDC/AGR-CFL/101707/2008, LISBOA-01-0145-FEDER-028760 and Grants to SS supported by Fundação para a Ciência e a Tecnologia (FCT/MCTES/PIDDAC, Portugal) through postdoctoral fellowship SFRH/BPD/108653/2015 and the work contract 2477 of the contract-program 12345/20181 defined by DL nr. Front. The standard curve was set that plot the threshold cycle (CT) with the plasmid DNA initial quantity, and the copy number in each sample was estimated making the correspondence between the CT, the quantity of plasmid and the mimicked copies of transgene.The expression of the Cast_Gnk2-like gene was analyzed by qPCR. Ecol. To test this, the number of survived days of transgenic and non-transformed lines was evaluated after plant inoculation with the oomycete. The State of the World’s Forests 2020: Forests, Biodiversity and People; FAO: Rome, Italy, 2020. Plant Cell 2020, 32, 1063–1080. [Google Scholar] [CrossRef] Figure 1. [Google Scholar] [CrossRef] [PubMed]Corredoira, E.; Merkle, S.A.; Martínez, M.T.; Toribio, M.; Canhoto, J.M.; Correia, S.I.; Ballester, A.; Vieitez, A.M. Non-zygotic embryogenesis in hardwood species. Res. Lett. Again, it was incubated overnight at 28 °C and with shaking (100 rpm) in darkness until an OD600 = 0.6 was reached. Plant Cell Tiss Organ Cult. Figure 4. 2013, 3, 3021. [Google Scholar] [CrossRef]Murashige, T.; Skoog, F. To this purpose, embryogenic cultures derived from adult trees of holm oak were transformed with the Cast_Gnk2-like gene and the disease response against this pathogen of the regenerated transgenic plants was evaluated.Proembryogenic masses of four embryogenic lines of holm oak, named Q8, Q10-16, E00 and E2, were transformed with the EHA105pK7WG2-Gnk2 strain. Progress in tissue culture, genetic transformation and applications of biotechnology to trees: An overview. A revised medium for rapid growth and bioassays with tobacco tissue culture. These transgenic lines were maintained by secondary embryogenesis, with subcultures every 6 weeks, following the conditions previously described in the Section 4.1.The presence of the transgenes (NPTII, GFP and Cast_Gnk2-like) was confirmed by amplifying its sequence by PCR. To date, there are no efficient chemical methods available for controlling this oomycete [9]. Subsequently, plants derived from the germination of non-transgenic and transgenic somatic embryos were transferred to these glass tubes. Cell 2013, 12, 194–203. After 14 weeks from the start of the transformation experiments, the efficiency of transformation, defined as the percentage of initial explants that show fluorescence (GFP+) was determined. Although the conversion frequencies were generally high, they varied considerably depending on the embryogenic line, probably because the number of gene copies, the mode of insertion and their position in the plant genome cannot be controlled [19].An in vitro assay was used to evaluate the tolerance of transgenic plants. After 14 weeks on selective medium, the transformation events were observed in somatic embryos of lines Q8 and E2 and a total of 4 transgenic lines were achieved. © 2022 by the authors. [Google Scholar] [CrossRef]Giri, C.C.; Shyamkumar, B.; Anjaneyulu, C. Int. Genomes 2014, 10, 977–988. [Google Scholar] [CrossRef]Xu, R.; Li, Q.Q. Protocol: Streamline cloning of genes into binary vectors in Agrobacterium via the Gateway®TOPO vector system. arabica [41]. Double selection via antibiotic resistance and expression of a GFP gene has also been successfully applied in other recalcitrant species, improving the selection process and reducing the escape number, selection time and antibiotic concentration [42,44].A series of molecular studies revealed expression of the Cast_Gnk2-like gene and estimated the copy number. 2017, 131, 321–333. Toribio (IMIDRA) for gift of the holm oak embryogenic lines Q8, E2 and E00. The Top 10 oomycete pathogens in molecular plant pathology: Top 10 oomycete plant pathogens. ilex, with a putative high level of similarity with the endogenous protein and a similar function, as the two species are closely related. [26] raised the possibility that binding of lectins to mannose residues of the Phytophthora cell wall may confer disturbance and disruption of the cell wall structure.The aim of the present paper was to investigate if the overexpression of the C. cinnamomi, then biochemical studies with the isolated protein will be essential to understand its action on oomycetes. (d) Plant derived from the germination of somatic embryo from a transformed line. In this respect, biotechnological tools such as somatic embryogenesis and genetic transformation provide a tremendous opportunity to improve tree characters [11,12,13,14]. Gastrodia anti-fungal protein from the orchid Gastrodia elata confers disease resistance to root pathogens in transgenic tobacco. [Google Scholar] [CrossRef] [PubMed]Song, P.; Cai, C.; Skokut, M.; Kosegi, B.D.; Petolino, J.F. Quantitative real-time PCR as a screening tool for estimating transgene copy number in WHISKERS™-derived transgenic maize. 2017, 8, 515. Holm oak somatic embryogenesis: Current status and future perspectives. cinnamomi [22]. cinnamomi strain and Ana Rita Carlos (Univ. Figure 1. Moreover, a strong GFP fluorescence signal was detected in holm oak somatic embryos and in different tissues derived from transgenic plants. [Google Scholar] [CrossRef]Chen, D.W.; Zentmyer, G.A. Production of sporangia by Phytophthora cinnamomi in axenic culture. The other two genotypes (Q10-16 and E00) did not render transformed explants. 2019, 38, 29–97. Chin. (e,f) GFP expression in a leaf (e) and in a root (f) excised from a transgenic plant and observed with ultraviolet light. Improving genetic transformation of European chestnut and cryopreservation of transgenic lines. Regeneration of Transgenic Plants by Agrobacterium-Mediated Transformation of Quercus ilex L. Because the strain may lose its ability to infect with time, before each infection experiment, it was reactivated, using the protocol established by [68]. For. For the qPCR expression analysis, Student´s t-test was applied.For the first time, the overexpression of Cast_Gnk2-like gene which codifies a Ginkbilobin-2 homologous domain gene reported with antifungal properties was attained. The PCR product was transferred into the pENTRTM/D-TOPO® vector (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Finally, all plants survived in control conditions (plants grown in the same in vitro tolerance assay medium but without the oomycete), which indicated that culture in liquid medium does not have a detrimental effect on the plants.Oak decline syndrome has been recognized as one of the most important ecological problems in Europe in the last few decades [5,28]. [24] report that Gnk2 functions as a lectin and hypothesize that its carbohydrate-binding properties are tightly related to its fungal activity. [Google Scholar] [CrossRef]Raveendar, S.; Premkumar, A.; Sasikumar, S.; Ignacimuthu, S.; Agastian, P. Plant Biol. 2010, 29, 513–522. After 30 min, the wash solution was removed by filtration, and the explants were transferred to proliferation supplemented with 300 mg/L CB and 75 mg/L kan. Holm oak is no exception and only one or two copies of the Cast_Gnk2-like gene have been detected.Although plant conversion from somatic embryos in many hardwoods is considered an important bottleneck [15], in the present study plants from four independent transgenic lines were successfully recovered by applying the protocols previously described for holm oak somatic embryos [9,50]. These authors contributed equally to this work. [Google Scholar] [CrossRef]Ruiz Gomez, F.J. Study of the Interaction between Root Rot Oomycetes and Quercus ilex L. Trees 2004, 18, 115–135. Upon thermal cycling, dissociation curves or melt curve analysis were used to analyze non-specific PCR products. Esp. 2017, 128, 447–455. Embryogenic cultures have been maintained by secondary embryogenesis with subcultures every 6 weeks, in proliferation medium that consisted of mineral medium SH [56], MS vitamins [57], 30 g/L sucrose and 6 g/L Plant Propagation Agar (PPA; Condalab, Madrid, Spain). [Google Scholar] [CrossRef]Corredoira, E.; Valladares, S.; Allona, I.; Aragoncillo, C.; Vieitez, A.M.; Ballester, A. Comparative transcriptomics-based selection of suitable reference genes for normalization of RT-qPCR experiments in drought-stressed leaves of three European Quercus species. As Q. Opin. [Google Scholar] [CrossRef]Yancheva, S.D.; Shlizerman, L.A.; Golubowicz, S.; Yabloviz, Z.; Perl, A.; Hanania, U.; Flaishman, M.A. The use of green fluorescent protein (GFP) improves Agrobacterium-mediated transformation of “Spadona” pear (Pyrus communis L.). Infection signs were more evident, and its progression was faster in non-transgenic plants than in transgenic plants, which appear completely fulminated between 4 and 6 days (Figure 4). After two weeks of culture in standard conditions, the explants were transferred to selection medium consisting of proliferation medium supplemented with 300 mg/L CB and 100 mg/L kan. [Google Scholar] [CrossRef]McGuigan, L.; Fernandes, P.; Oakes, A.; Stewart, K.; Powell, W. All amplification reactions were carried out in an MJ Mini thermal cycler (BioRad, Hercules, CA, USA), using a reaction volume 50 µL. Extremadura) for providing P. Recent progress in Agrobacterium tumefaciens-mediated genetic transformation of somatic embryos of different species in the Fagaceae family, including holm oak, have provided the opportunity to produce transgenic genotypes [16,17,18,19]. Natl. Figure 4. The cDNA sequence encoding Cast_Gnk2-like was amplified by PCR applying the proofreading ADN polimerase AccuPrime™ Pfx (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was extracted from non-transformed and putatively transformed lines using somatic embryos at early cotyledonary stage and the Kit Qiagen RNeasy Plant Mini (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Negative controls (explants not transformed but cultured in selection medium) necrotized and died without forming callus or somatic embryos. LineCT MeanCT Standard DeviationEstimated Copy Number 1Q8-WT27.270.970Q8-Gin121.550.611Q8-Gin220.510.272E2-WT27.351.350E2-Gin121.690.411 Table 3. DNA extraction from non-transgenic and transgenic somatic embryos was carried out using the Kit REALPURE (Durviz, Valencia, Spain) following the instructions of the manufacturer and prior homogenization of the plant material (100 mg) in mortars with liquid nitrogen. In the present study for Q. Mother trees used as source of initial explants to induce embryogenic lines were growing in two locations of Spain: genotypes E in Alcala de Henares and genotypes Q in Toledo for more details see [50,55]. The PCR amplification products were resolved on 1.5% gels agarose (Ultra Pure Agarose, Invitrogen, Waltham, MA, USA), in TBE 1X (Duchefa, Haarlem, the Netherlands) and 1% of RedSafe (iNtRON Biotechnology, Sangdaewon-Dong, Korea) dye. Acad. Germination and infection survival data were tested for homogeneity of variance (Levene Statistics). [Google Scholar] [CrossRef]Yang, K.; Rong, W.; Qi, L.; Li, J.; Wei, X.; Zhang, Z. DNA concentration was quantified on a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cinnamomi inoculation, and a set of candidate genes of resistance was selected [21]. ilex DNA to simulate 1, 2, 5 and 10 copies of transgene. Somatic Embryos with the Gene CsTL1. 2019, 10, 239. The arcsine square-root transformation was applied to proportional data prior to analysis. [Google Scholar] [CrossRef]Marum, L.; Miguel, A.; Ricardo, C.P.; Miguel, C. [Google Scholar] [CrossRef]Mou, S.; Meng, Q.; Gao, F.; Zhang, T.; He, W.; Guan, D.; He, S. Rev. [Google Scholar]Narváez, I.; Pliego Prieto, C.; Palomo-Ríos, E.; Fresta, L.; Jiménez-Díaz, R.M.; Trapero-Casas, J.L.; Lopez-Herrera, C.; Arjona-Lopez, J.M.; Mercado, J.A.; Pliego-Alfaro, F. The bacteria were eliminated by immersion of explants in a washing solution which consisted of sterile water with carbenicilin (300 mg/L; CB). Effect of genotype on percentage of kanamycin-resistant explants, transformation efficiency and selection efficiency following 14 weeks of culture in selection medium. cinnamomi has not yet been checked.Although the number of transformation systems has increased greatly in recent years, production of transgenic plants remains difficult and is limited to a small number of species and genotypes [36,37]. [Google Scholar] [CrossRef]Ribas, A.F.; Dechamp, E.; Champion, A.; Bertrand, B.; Combes, M.C.; Verdeil, L.; Lapeyre, F.; Lashermes, P.; Etienne, P. 2000, 279, 407–411. [Google Scholar] [CrossRef]Kamoun, S.; Furzer, O.; Jones, J.D.G.; Judelson, H.S.; Ali, G.S.; Dalio, R.J.; Roy, S.G.; Schena, L.; Zambounis, A.; Panabières, F.; et al. [Google Scholar] [CrossRef] [PubMed]Miyakawa, T.; Hatano, K.-I.; Miyauchi, Y.; Suwa, Y.-I.; Sawano, Y.; Tanokura, M. Cult. After 10 weeks on the selection medium, the percentage of kanamycin (kan)-resistant explants was recorded. Transformation results were analyzed by Chi-Square test (χ2). [Google Scholar] [CrossRef]Leclercq, J.; Lardet, L.; Martin, F.; Chapuset, T.; Oliver, G.; Montoro, P. Agric. [Google Scholar]Rial, C.; Barros, A.; Salinero, C.; Mansilla, J.P.; Pintos, C.; Cuenca, B. McGuigan et al. [Google Scholar] [CrossRef]Matzke, A.J.; Matzke, M.A. Position effects and epigenetic silencing of plant transgenes. Gnk2 has a plant-specific cysteine-rich motif DUF26 (domain of unknown function 26, also known as stress-antifungal domain: PF01657) which belongs to cysteine-rich receptor-like kinases (CRKs) not showing any similarity with other known antimicrobial proteins. Plant defense and antimicrobial peptides. Academic Editor: Keunsub Lee Plants 2022, 11(3), 304; Received: 25 December 2021 / Revised: 19 January 2022 / Accepted: 20 January 2022 / Published: 24 January 2022 Holm oak is a key tree species in Mediterranean ecosystems, whose populations have been increasingly threatened by oak decline syndrome, a disease caused by the combined action of Phytophthora cinnamomi and abiotic stresses. [Google Scholar] [CrossRef]Kimura, S.; Hunter, K.; Vaahtera, L.; Tran, H.C.; Citterico, M.; Vaattovaara, A.; Rokka, A.; Stolze, S.C.; Harzen, A.; Meißner, L.; et al. LineOnly RootConversion (Shoot + Root)(%)RL (mm)(%)RL (mm)SL (mm)NLLines Q8 Q8-WT58.0 ± 6.852.5 ± 5.938.9 ± 5.154.9 ± 7.510.8 ± 0.97.6 ± 0.8Q8-Gin133.3 ± 14.788.8 ± 9.952.8 ± 16.469.7 ± 1.811.6 ± 1.27.9 ± 0.2Q8-Gin211.1 ± 3.2105.0 ± 18.469.5 ± 10.078.3 ± 7.412.1 ± 0.99.5 ± 0.7Q8-Gin347.2 ± 12.158.9 ± 7.430.6 ± 4.742.2 ± 8.510.1 ± 1.99.5 ± 2.1ANOVA Insnsnsp < 0.05nsnsLines E2 E2-WT55.6 ± 10.148.5 ± 5.638.9 ± 10.171.1 ± 13.116.7 ± 1.88.0 ± 1.3E2-Gin18.3 ± 3.4100.0 ± 30.122.2 ± 8.562.3 ± 9.215.5 ± 0.44.8 ± 0.8ANOVA Ip < 0.05nsnsnsnsns Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations. DNA was extracted as described in the Section 4.5.1, from non-transgenic and transgenic somatic embryos and amplified independently to achieve three biological replicates for each line. [Google Scholar] [CrossRef] [PubMed]Corcobado, T.; Cubera, E.; Juárez, E.; Moreno, G.; Solla, A. Based on our data, we suggest that the Cast_Gnk2-like gene is a promising gene for further functional analysis studies to improve plant disease tolerance. After the electrophoresis is complete, the gels were visualized with a transilluminator equipped with an imaging program (Quantity One 1-D Analysis Software, BioRad, Hercules, CA, USA).The number of copies of the Cast_Gnk2-like gene inserted in each putative transgenic line was estimated by quantitative real-time PCR (qPCR). Genotype significantly affected this parameter (p ≤ 0.05), and new somatic embryos and/or nodular structures were only observed on lines Q8 and E2, with Q8 being the highest percentage (5%) (Table 1; Figure 1a). Therefore, Cast_Gnk2-like gene may prevent pathogen growth either by its chemical properties or by inducing HR-related cell death. Biol. Transformation of American Chestnut (Castanea dentata (Marsh.) Borkh) Using RITA® Temporary Immersion Bioreactors and We Vitro Containers. Plant defensins. All transgenic lines derived from genotype Q8 survived longer than the untransformed control line, but only the Q8-Gin1 line (10.35 days) showed significantly (p < 0.05) higher survival ability than the control line (4.46 days) (Figure 5). Expression analysis of Cast_Gnk2-like gene in somatic embryos of holm oak. Figure 3. P. On other hand the Tm of the WT amplicon (84 °C) was distinct from the Tm of the transformed lines amplicon (80 °C) (Online resource 3). 2021, 21, 382. Dissociation curves were used to analyze non-specific PCR products. 2014, 192–193, 1–8. cinnamomi is one of the most destructive and widely spread oomycetes throughout the world, and it has been associated with the death of numerous different plant species, including many woody species [8]. Kan-resistant embryos were isolated and transferred to medium proliferation supplemented with a higher concentration of kan than the selection medium (125 mg/L) and were cultivated for 4 more weeks in order to increase the selective pressure and confirm its resistance to kan. The vector pK7WG2D (Department of Plant Systems Biology, VIB University of Ghent, Belgium) also includes the selectable marker gene neomycin phosphotransferase (NPTII) and the reporter gene for green fluorescence protein (GFP). This is due to the copy number as well as to other factors such as the integration site and the presence of incomplete copies [46,47]. [Google Scholar] [CrossRef]Mélida, H.; Sandoval-Sierra, J.V.; Diéguez-Uribeondo, J.; Bulone, V. The findings confirm the significant increase in survival (days) in two lines (Q8-Gin1 and E2-Gin1), both of which have one copy of the Cast_Gnk2-like gene and display greater expression of the transgene than the respective wild-type genotypes. [Google Scholar] [CrossRef]Martínez, M.T.; San-José, M.C.; Vieitez, A.M.; Cernadas, M.J.; Ballester, A.; Corredoira, E. Cox et al. 2013, 169, 92–99. Plant Methods 2008, 4, 4. Cienc. Asterisks indicate significant differences in the expression when compared to WT with p < 0.05 (t-test). After 3 days of culture, a colony isolated from this dish was inoculated in 2 mL of liquid LB medium supplemented with 50 mg/L kan. After this time, the lines putatively transformed were transferred to proliferation medium without antibiotics, to remove selection pressure and promote growth and multiplication of the lines. 2011, 11, 92. New Agrobacterium helper plasmids for gene transfer to plants. The presence of the Cast_Gnk2-like gene on transgenic embryos was verified by PCR, and the number of transgene copies and gene expression was estimated by qPCR. These studies have investigated overexpression of the pathogenesis-related thaumatin-like protein in order to induce tolerance to P. cinnamomi observed in in vitro transformed Cast_Gnk2-like holm oak plants suggests the involvement of the Gnk-2 like protein and indicates the need for further studies to clarify the action of the protein against oomycetes.For the genetic transformation of holm oak, four embryogenic lines were used: E2, Q8, E00 and Q10-16. Plant Sci. Genomes 2019, 15, 38. After four weeks of cultivation under these conditions (14 weeks in total), the efficiency of transformation based on the embryo fluorescence of kan-resistant explants (Figure 1b,c) was calculated, and values of 2.5% were obtained in both genotypes (Table 1). A cysteine-rich receptor-like protein kinase CaCKR5 modulates immune response against Ralstonia solanacearum infection in pepper. Three biological replicates per line were considered. Specific oligos designed for the promoter are described in Online resource 2. Selection efficiency ranged from 50 to 75%, and the highest figure for selection efficiency (75%) was found in genotype E2 (Table 1).To establish putative transgenic lines, an embryo was isolated at the cotyledonary stage that showed distinct GFP expression uniformly distributed over the entire embryo surface, discarding those that were not totally fluorescent. Reference Gene Selection for Quantitative Real-time PCR Normalization in Quercus suber. [Google Scholar] [CrossRef]Available online: (accessed on 22 November 2021).Serrazina, S.; Santos, C.; Machado, H.; Pesquita, C.; Vicentini, R.; Pais, M.S.; Sebastiana, M.; Costa, R. Stability of the extracted RNA was verified by observing a double band in gels of 1.2% agarose with formaldehyde, and the concentration was quantified with a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Specific primers for Cast_Gnk2-like are described in Online resource 2. The tolerance of plants against the oomycete was evaluated as the number of days of survival in an incubation period of 15 days. Plant Signal. This was expected, as the transgene Cast_Gnk2-like is guided by the constitutive promoter CaMV35S.In the transformed lines, Q8-Gin1, Q8-Gin3 and E2-Gin1 had significantly (p ≤ 0.05) higher expression levels of Cast_Gnk2-like when compared to the non-transformed genotypes, indicating an overexpression of the Cast_Gnk2-like gene. 2000, 11, 298–302. Survival ability of transformed and non-transformed (WT) holm oak plants derived from genotypes Q8 and E2 after 15 days of infection with P. [Google Scholar]Guzmán-Álvarez, J.R. The image of a tamed landscape: Dehesa through History in Spain. Cell 2005, 37, 2239–2253. 2017, 7, 239. The amplification programs used, the sequence of the primers as well as the size of the generated fragment are specified in Online resource 2. Estimated copy number in holm oak somatic embryogenic lines transformed with Cast_Gnk2-like gene. Manag. Subsequently, only transgenic lines that produce the best results can be multiplied and acclimatized, thereby reducing the time required and material used. [Google Scholar] [CrossRef] [PubMed]Yan, J.; Yuan, S.S.; Jiang, L.L.; Ye, X.J.; Ng, T.B.; Wu, Z.J. Plant antifungal proteins and their applications in agriculture. Table 1. 2015, 16, 413–434. 2016, 7, 938. Q8-Gin1 is the line with the highest transgene expression. Meteorol. 2015, 194, 181–187. Plant Cell Biotechnol. Elapsed this time, somatic embryos were cultured in glass jars 500 mL with 70 mL of germination medium. [Google Scholar] [CrossRef]Hood, E.E.; Gelvin, S.B.; Melchers, L.S.; Hoekema, A. [Google Scholar] [CrossRef]Cano, V.; Martínez, M.T.; Couselo, J.L.; Varas, E.; Vieitez, F.J.; Corredoira, E. Regeneration performance with only root development and conversion into plantlets (simultaneous root and shoot development) of different holm oak transformed and untransformed (WT) lines after 8 weeks on germination medium. 3 Biotech. Although ginkbilobins do not resemble other pathogenesis-related proteins, they display homology to the extracellular domain of plant cysteine-rich receptor-like kinases, which explains their role [34]. crenata Ginkbilobin-2 homologous domain gene (Cast_Gnk2-like) enhances tolerance to P. In this chamber, light was supplied by tubes white light fluorescent lamps (Mazdafluor 7D TF 36 w/LJ) with a photon flux density of 50–60 $\mu mol \cdot m^{-2} \cdot s^{-1}$.The chestnut Ginkbilobin2 homologous domain gene (Cast_Gnk2-like) identified by Serrazina et al. Proc. Low transformation rates have also been reported for other recalcitrant species, cultivars and genotypes. It can be concluded that the infection in liquid medium method is very useful for the rapid evaluation of several transformed lines, enabling initial selection of those lines showing the greatest tolerance to the pathogen. The reaction is composed of 250–500 ng of genomic DNA, 1 µL Cl2Mg, 0.2 µL Taq DNA Polymerase (Qiagen, Hilden, Germany), 2.5 mM of dNTPs and 15 µM of primers. Ginkbilobin proteins may prevent the growth of pathogens by chemical reactions or by inducing hypersensitive response-related cell death. Thisprotein, named ginkbilobin has exhibited potent antifungal activity against diverse fungi, including Botrytis cinerea, Mycosphaerella arachidicola, Fusarium oxysporum, Rhizoctonia solani and Coprinus comatus [29,30]. Expression in transformed lines was calculated relative to the respective non-transgenic lines (WT), and normalized to EF1α and TUB reference genes. Mou et al. This species contributes to the direct production in dehesas, providing acorns, wood, comestible fungi, pollen and other products consumed by humans and animals [6]. Selection of Castanea sativa Mill., for resistance to Phytophthora cinnamomi: Micropropagation and testing of selected clones. [Google Scholar] [CrossRef] [PubMed]Barra-Jiménez, A.; Blasco, M.; Ruiz-Galea, M.; Celestino, C.; Alegre, J.; Arrillaga, I.; Toribio, M. Among oak species, holm oak is considered one of the most susceptible to the syndrome. Curr. Transgenic Res. In line E2, the conversion frequencies were higher in the non-transformed line (38.9%) than in E2-Gin1 (22.2%), but no significant differences were detected between transgenic and wild type material (Table 3). Instead, qPCR was chosen as it is sensitive and permits specific quantification in a high number of samples with low DNA quantities [27,45]. Crit. A Petri dish with solid LB medium (1.5% agar) was inoculated with this bacterial suspension, and the dish was incubated at 28 °C in darkness. [Google Scholar] [CrossRef]Castro, M.S.; Fontes, W. Although the transformation efficiency was low (2.5%), it was similar to the values reported in a previous paper (1.66–3.33%) on holm oak genetic transformation [18]. Fluorescence was detected in shoots and roots of transgenic plantlets (Figure 1e,f), indicating stable transformation of these lines. Hortic. The embryos were maintained under standard conditions, with periodic subcultures every two weeks to this selection medium, for 10 weeks from the beginning of the experiment.For each embryogenic line, 120 explants (12 Petri dishes with 10 explants) were used. As in Song et al. [Google Scholar] [CrossRef]Gresshoff, P.M.; Doy, C.H. Development and differentiation of haploid Lycopersicon esculentum. Proembryogenic masses (PEMs) isolated from four embryogenic lines (Q8, E2, Q10-16 and E00) were used as target explants. (b,c) GFP expression in transformed holm oak somatic embryos observed under white light (b) and the same embryos observed with ultraviolet light (c). 2013, 116, 141–151. 2010, 45, 411–418. The pH was adjusted to 5.6 before autoclaving at 115 °C for 20 min. [Google Scholar] [CrossRef]Martínez, M.T.; Arrillaga, I.; Sales, E.; Pérez-Oliver, M.A.; González-Mas, M.C.; Corredoira, E. Bot. The resistant embryos in both lines appeared after 8 weeks of culture in selection medium, and in general, the target explants presented better appearance and lower level of necrosis in these lines than the initial explants of lines E00 and Q10-16. 2009, 28, 1385–1397. A schematic representation of T-DNA region of the plasmid pK7WG2D-Gnk2. Genomes 2015, 11, 6. [Google Scholar] [CrossRef] [PubMed]Parmar, N.; Singh, K.; Sharma, D.; Singh, L.; Kumar, P.; Nanjundan, J.; Khan, Y.J.; Chauhan, D.K.; Thakur, A.K. Genetic engineering strategies for biotic and abiotic stress tolerance and quality enhancement in horticultural crops: A comprehensive review. cinnamomi, a mycelium disc with sporangia developed as described in previous section were placed on the bottom of each glass tube with 15 mL of GD liquid medium supplemented with sucrose 3% and 0.1 mg/L BA [68,70]. Once RNA was extracted, it was treated with the RNase-free DNase Set (Qiagen, Hilden, Germany) to eliminate a possible DNA contamination. Asterisks indicate significant differences in the survival when compared to WT with p < 0.05 (Tukey’s HSD test). To normalize expression data, Elongation factor 1 alpha (EF1α) [62] and β-Tubulin (TUB) [63] were used and the oligos, designed for species within Quercus genera, are described in Online resource 2. Finally, selection efficiency was estimated as the percentage of kan-resistant explants that also developed GFP-positive embryos.From each GFP + explant, a single embryo at cotyledonary stage was isolated and cultured on selective medium for at least four subcultures to establish putative transgenic embryogenic lines. Ph.D. Thesis, Universidad de Córdoba, Córdoba, Spain, 2018. A key factor in achieving genetic transformation in this species is the use of PEMs as target explants.

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