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Root Rot Resistance Locus *PaLAR3* Is Delivered by Somatic Embryogenesis (SE) Pipeline in Norway Spruce (*Picea abies* (L.) Karst.)

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Abstract: Research Highlights: The Norway spruce somatic embryogenesis (SE) pipeline is suitable for multiplication of material with root rot resistance traits. Background and Objectives: *Heterobasidion* root rot is the economically most severe forest pathogen in Europe, reducing the benefit of planting elite forest material. In this study, the SE-propagation ability of elite Norway spruce material carrying root rot resistance traits was studied. Materials and Methods: We analyzed the presence of the root rot resistance locus *PaLAR3B* among 80 Finnish progeny-tested Norway spruce plus-trees used for SE-plant production as well as in 241 SE lines (genotypes) derived from them. Seven full-sib families with lines having either AA, AB, or BB genotype for *PaLAR3* locus were further studied for their SE-plant propagation ability. Results: The results indicate that 47.5% of the studied elite trees carry the *PaLAR3B* allele (45% are heterozygous and 2.5% homozygous). The resistance allele was present among the SE lines as expected based on Mendelian segregation and did not interfere with somatic embryo production capacity. All embryos from *PaLAR3* genotypes germinated well and emblings were viable in the end of first growing season. However, in three families, *PaLAR3B* homo- or heterozygotes had 23.2% to 32.1% lower viability compared to their respective hetero- or *PaLAR3A* homozygotes. Conclusions: There is no trade-off between root rot resistance locus *PaLAR3B* and somatic embryo production ability, but the allele may interfere with Norway spruce embling establishment.

Keywords: genomic selection; somatic embryogenesis; root rot resistance; vegetative propagation

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1. Introduction

Norway spruce (*Picea abies* (L.) Karst.) is one of the most important conifers in Europe, providing valuable raw material for forest industries [1]. The stem and root rot caused by members of the *Heterobasidion annosum s.l.* species complex is considered the most severe fungal forest disease occurring in Norway spruce in Europe. *H. annosum s.l.* incidence has been projected to increase in the future, thus threatening the expected productivity increase associated with planting elite forest regeneration materials [2,3].

The Norway spruce genome harbors genetic variability in root rot resistance which could be utilized in breeding for more resistant forest regeneration material [4]. Lind et al. [5] detected 13 quantitative trait locus (QTL) regions in the Norway spruce genome controlling resistance to *Heterobasidion parviporum*. By now, variation in one gene, leucoanthocyanidin reductase 3 (*PaLAR3*) comprising one of the QTLs, is known to a play role in root rot resistance [6].

PaLAR3 encodes an enzyme that catalyzes the formation of the 3-flavanol (+)-catechin associated with increased resistance against pathogens and pests [7,8]. The allele *PaLAR3B* that confers higher resistance to the Norway spruce when present, acts as a dominant allele. The presence of the particular allele is associated with higher (+)-catechin content and a 27% reduction of *H. parviporum* in Norway spruce [6] making it an attractive trait for forest tree breeding.

Conventional breeding is a time-consuming process, especially in forest trees, due to their large size and long lifespan [1,3,9]. Moreover, fluctuant flowering of spruce may cause intermittent shortage of high-quality seed material for forest regeneration. In Finland, over 100 million spruce plants are needed every year for forest regeneration purposes [10]. The availability of high-quality forest re-planting material can be supported by biotechnology solutions such as vegetative propagation by somatic embryogenesis (SE) combined with cryopreservation of the best material [11].

Somatic embryogenesis enables fast and efficient multiplication of specific genotypes with desired characteristics, e.g., fast growth, wood quality, ornamental value, or insect resistance traits [12,13]. The valuable genotypes can be stored by cryopreservation to avoid ageing and are ready for re-multiplication when needed. SE has been adopted for commercial spruce regeneration material production in several places around the world [13,14]. The SE-production of high-quality spruce genotypes would be even more valuable if they would carry resistance traits, such as *Heterobasidion* resistance. However, SE is a delicate process affected by many genetic and physiological factors through complex regulatory networks that still remain partly unknown [15].

Production of phenolic compounds involved in resistance traits has been seen as harmful for in vitro cultures by affecting the resource allocation in growing tissues and altering, or interacting, with the SE-production process. In white spruce, *Picea glauca*, it has been suggested that activation of genes involved in biotic defense may, e.g., suppress SE-induction in shoot primordial explants [16]. For instance, Businge et al. reported accumulation of (+)-catechin in a cell line producing aberrant embryos, but not in the cell line producing normal embryos, during the late embryogeny and maturation phases [17].

As the resistance allele *PaLAR3B* is associated with higher (+)-catechin accumulation in Norway spruce, the aim of the present study was to analyze whether the presence of this allele, *PaLAR3B*, interferes with SE-plant production. We hypothesize that in the case where there is no trade-off between resistance allele and SE-production, then resistance allele delivery would not differ from what is expected according to Mendelian inheritance and there are no differences in embryo production capacity (E/gFW) and embling (SE-propagated plant) performance in nursery conditions.

This is the first study of SE-propagation of forest regeneration material that carries resistance traits. In the present study, the resistance allele *PaLAR3B* frequency among Finnish plus-trees used for SE-propagation as well as the allele delivery through SE-propagation process from elite trees to their SE-progeny is analyzed.

2. Materials and Methods

2.1. Plant Material

Eighty Finnish Norway spruce plus-trees used in crosses and SE-initiations from 2011 to 2019 and 241 SE lines (genotypes) from seven full-sib families derived from 14 of the analyzed plus-trees (19–67 SE lines per family) were used in the study.

2.2. Genotyping of the PaLAR3 Locus

DNA was extracted from buds or needles of plus-trees and from embryogenic tissues (ET) or somatic embryos of SE lines with E.Z.N.A® SP Plant DNA Kit, Omega Bio-tek®.

To allow for fast, single-tube screening of the *PaLAR3* locus, primers specific for one locus and two alleles were designed against the genomic sequence of *PaLAR3* (KX574230.1 and KX574229.1). For detection of *PaLAR3* alleles in a genotyping assay, 10–40 ng genomic DNA per sample was used in 10 µL PCR reactions with 1× Inhibitor Resistant Genotyping

PCR ReadyMix (PIR00, Sigma-Aldrich, St. Louis, MI, USA). In each reaction, 0.4 µM of locus-specific LAR_ComA and 0.2 µM of each allele-specific primer (Table S1) were included. The PCR conditions were 94 °C for 5 min; followed by 35 cycles of 30 s at 94 °C, 30 s at 54 °C, 30 s at 72 °C; and final elongation for 7 min at 72 °C. Genotypes were scored based on the presence or absence of a 110 and 201 bp band, representing *PaLAR3A* and *PaLAR3B*, respectively, after agarose gel electrophoresis.

2.3. SE-Propagation

2.3.1. SE-Initiations

The SE lines were initiated between 2011 and 2019 using medium and methods developed by Klimaszewska et al. [18] as described by Varis et al. [19]. In short, immature zygotic embryos originating from controlled crosses of progeny-tested plus-trees from Southern Finland were placed on modified Litvay's medium (mLM) (half-strength macroelements, 1% sucrose (*w*/*v*)), 10 µM 2,4-dicholophenoxyacetic acid (2,4-D) and 5 µM 6-benzyladenine (BA), pH 5.8, gelled with Phytagel (4 g/L). After autoclaving, 500 mg/L of filter-sterilized L-glutamine was added to the medium cooled to 60 ° C. Petri dishes (9 cm in diameter) were filled with 20 mL of medium. Cultures were kept in the dark (24 ° C) for 2 to 8 weeks without subculturing until embryogenic tissue (ET) started to proliferate. Established ETs were subcultured bi-weekly by transfer to fresh Petri plates with the same medium. Initiation data was analyzed from 55 full-sib families (5–540 initiation attempts, i.e., explants per family, 8588 initiation attempts in total resulting in 5393 successful SE-initiations (Table S2).

2.3.2. Cryopreservation

To avoid ageing of the ETs, the cultures were preserved in liquid nitrogen (LN, −196 °C) four to ten weeks after establishment. Cryopreservation and thawing were conducted according to the slow cooling method of Varis et al. 2017 [19]. In short, for preconditioning, fresh ET clumps were cultured onto solid mLM media with increasing sucrose content (0.1 and 0.2 M sucrose for 24 h for each concentration). After preconditioning, 200 mg ET was placed in sterile cryovials containing 400 µL liquid mLM medium with 0.4 M sucrose without plant growth regulators (PGR) or glutamine. Cryovials were placed on thermoconductive racks (CoolRack CFT30) precooled to −20 °C. To each cryovial, 200 µL prechilled cryoprotectant PGD solution (polyethylene glycol 6000, glucose, and DMSO 10% *w/v* each) was added twice during 30 min. Thereafter, cryovials were incubated 30 min in the thermoconductive racks and cooled at 0.17 °C/min to −38 °C in a programmable cooling device (Planer, Kryo 10 Series III, Planer Products, Middlesex, UK) followed by immersion in LN.

Regeneration of ETs was started by thawing the vials in a water bath at +37 °C for 2 min. After thawing, the tissues were poured on sterilized filter paper (Whatman #2, Whatman International Limited, Kent, England) placed in a Büchner funnel. The cryoprotectant was drained off by suction, and the tissues were washed with 2 mL liquid mLM medium (0.4 M sucrose without PGR or glutamine). The filter papers with samples were placed on solid mLM medium with sucrose content of 0.2 M and transferred every 24 h onto media with decreasing sucrose concentration (0.1 M then 0.03 M).

2.3.3. Embryo Production Capacity and Embling Viability

Next, the SE lines were studied for their ability to produce somatic embryos. From each SE line, 3–5 maturation plates were made using filter paper method modified from Lelu-Walter and co-workers [14].

For maturation approx. 150 mg ET was suspended in 3 mL liquid mLM without PGR and poured onto filter paper (Whatman #1) placed in a Büchner funnel. The liquid was drained off by suction and the filter paper with ET was placed on mLM medium with 30 µM abscisic acid (ABA) and 0.2 M sucrose, gelled with 6 g/L Phytagel [20]. After eight weeks of maturation, the number of cotyledonary embryos having at least four cotyledons

per gram of fresh weight (E/gFW) was recorded. Overall, E/gFW was analyzed from 191 SE lines from seven SE families.

Cold storage, germination, and transplanting of in vitro germinated embryos to peat-based growth substrate was carried out according to Tikkinen et al. (2018) [20]. In short, the method includes cold storage of the cotyledonary embryos in +2 °C on filter papers in the maturation plates; embryos on semisolid germination medium for one week in vitro under LED lights in spectrum AP67 (Valoya) under 18:6 photoperiod at 20 °C in increasing light intensity (5, 50, and 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ used in time proportions of 43.0%, 28.5%, and 28.5%,); transplanting to substrate using the pricking out method [21,22]. From each SE line, 5 to 81 emblings were germinated (94 SE lines from four families: 18–32 SE lines/family, total 3450 embryos). The number of embryos going into germination was adjusted so that they could be transplanted in full rows in Plantek 81f containers. No more than 81 embryos (one full pl 81f container) were germinated even if there were more embryos available.

All embryos which were put to germination were transplanted into nursery. Transplanting of in vitro germinated embryos was carried out in a greenhouse of a commercial forest nursery, where the emblings were grown together with Norway spruce seedlings with the same growing protocols as used in natural light. To maintain ambient and edaphic conditions suitable for Norway spruce seedlings suggested by Landis et al. [23] and Rikala [22], identical measures between seedlings and SE-plants were taken after the in vitro germination and transplanting to peat. Viability after first growing season in the nursery (% of emblings alive in the end of first growing season from transplanted embryos) was analyzed from 49 SE lines (from four families: 3–9 SE lines/family, 6–36 emblings/SE line, total 1498 emblings).

2.4. Statistical Analyses

The initiation success of different *PaLAR3* parent cross genotypes and embling viability was analyzed in contingency tables by Chi-squared test of independence using the Vcd package [24]. Factors affecting initiation results were further analyzed with logistic regression using IBM SPSS Statistics 26. The effects of *PaLAR3* genotype of each parent together and individually, progeny genotype of *PaLAR3* (AA; AA or AB; AB or BB) on the binary response (whether initiation from a single explant was successful or not), in addition to the effect of initiation year and full-sib family were investigated in the initiation data from the years 2011 to 2015. Data from 2019 were excluded from the analyses because recording of successful initiations was stopped after the predetermined number of SE lines per full-sib family had been achieved. Only full-sib family improved the model (above 1% increase in the cases predicted correctly by the model) of the all tested factors (other factors did not improve the model at all). For this reason, only full-sib family was selected to final model of the tested factors.

The observed allele delivery through SE-pipeline was analyzed against expected Mendelian segregation ratio (1:1 for homozygote × heterozygote cross and 1:2:1 in a case both parents were heterozygous) by Chi-squared analysis in each family. Embryo production capacity (E/gFW) was analyzed by Kruskal–Wallis test followed by Dunn's test [25] using dunn.test package for R [26] (version 1.3.6). Embling viability was analyzed in contingency tables by Chi-squared test of independence using the Vcd package [24]. All statistical analyses were performed using statistical software R [27]. For visualization, package ggplot2 [28] and ggforce [29] were used.

3. Results

3.1. Parent Tree Genotyping

The results indicate that 47.5% of the 80 Finnish plus-trees used for SE-initiations carry the *PaLAR3B* allele (45% are heterozygous and 2.5% homozygous) (Table 1). Thus, the allele frequency of the dominant *PaLAR3B* allele among the 80 parental trees used for SE-initiations between 2011 and 2019 was 0.25.

Table 1. *PaLAR3* allele distribution among 80 Finnish Norway spruce (*Picea abies*) plus-trees used for SE-initiations from 2011 to 2019 in Finland.

Allele	*n*	%
AA	42	52.5
AB	36	45
BB	2	2.50

3.2. SE-Initiations

SE initiations were successful in all tested crosstypes, varying from 63.5% to 85.4% within each crosstype in years 2011–2015 (Figure 1). There were significant differences in initiation percentages between different plus-tree crosstypes based on *PaLAR3* genotypes (X^2 = 188.87, df = 5, $p < 0.001$). However, when other factors affecting initiation success (year and family) were included in the analyses, family become the most significant factor affecting initiation results, whereas *PaLAR3* genotype did not have an effect on the initiation results (Table S3).

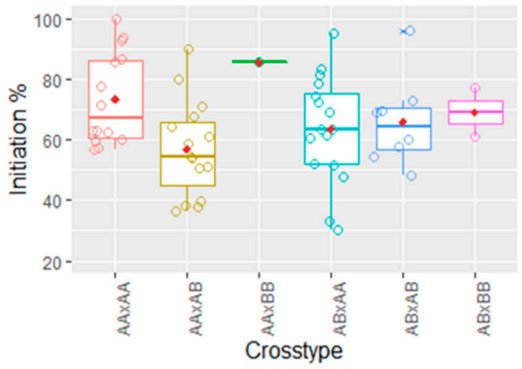

Figure 1. Mean SE-initiation % of different parent *PaLAR3* genotypes from 2011 to 2015. Each circle denotes initiation % of one full-sib SE-family.

3.3. PaLAR3B Allele Delivery through SE-Pipeline

Among the 241 analyzed SE lines resulting from crosses between 14 parents with different *PaLAR3* genotypes (AA × AB, AB × AA, AB × BB or AB × AB), 122 were hetero- and 73 homozygous for the dominant *PaLAR3B* allele (Table 2). In most families, the *PaLAR3B* allele was successfully delivered to the SE-progeny as expected according to the Mendelian inheritance rule. One family deviated from the expected genotype ratios based on the parental genotypes. The SE-progeny of E9 × E3231 deviated from the expected 1:1 ratio at $p = 0.03$ (X^2 = 5, df = 1, Table 2).

Table 2. The *PaLAR3B* allele delivery through the SE-pipeline in seven Finnish elite tree SE-families.

Family	Parent *PaLAR3* Genotype	SE-Progeny *PaLAR3* Genotype (*n* of SE Lines)			
		AA	AB	BB	Total
E462 × E64	AA × AB	14	9	-	23
E242 × E222	AB × AA	9	10	-	19
E9 × E3231	AB × AA	9	21 [a]	-	30
E46 × E3222	AB × BB	-	17	19	36
E9 × E1361	AB × BB	-	34	33	67
E2515 × E1204	AB × AB	5	12	5	22
E81 × E3224	AB × AB	10	18	16	44
	Total:	45	122	73	241

[a] Observed frequency differs from expected 1:1 ratio at $p < 0.5$.

3.4. Embryo Production Ability

Mean embryo production of different *PaLAR3* genotypes representing seven families was 86.6, 93.2, and 101.3 E/gFW for *PaLAR3* AA, AB, and BB genotypes, respectively (Figure 2A). Within most families, the embryo production potential did not differ between *PaLAR3* genotypes, but in three families, a variation in embryo production ability based on the *PaLAR3* genotype was detected (Figure 2B). In the SE-progeny from E462 × E64 *PaLAR3B* heterozygotes had significantly lower embryogenic potential than *PaLAR3A* homozygotes (72.1 vs. 104.7 E/gFW, $p < 0.05$). Among SE-progeny from E81 × E3224 *PaLAR3B* homozygotes produced less embryos (25.7 E/gFW) compared to their heterozygotes (65.1 E/gFW, $p < 0.01$) while the progeny from E46 × E3222 *PaLAR3B* homozygote embryo production ability was higher (137.8 E/gFW) compared to their heterozygotes (100.1 E/gFW, $p < 0.05$).

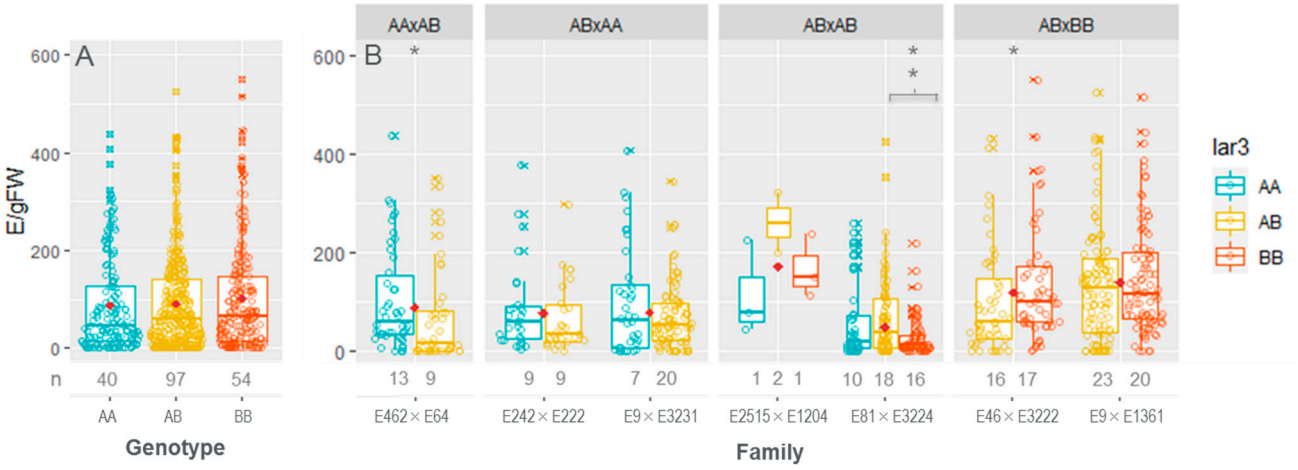

Figure 2. Embryo production capacity (embryos per gram fresh weight; E/gFW) of different *PaLAR3* genotypes (**A**) of seven Norway spruce full-sib SE-families (**B**), with the number of SE lines given for each allelic combination (*n*). Asterisks denote differences between genotypes within each family (* $p < 0.05$, ** $p < 0.01$).

3.5. Embling Viability

All embryos selected for in vitro germination tests germinated independent of their *PaLAR3* genotype and were transplanted into a greenhouse. Viability of the produced emblings was analyzed at the end of the first growing season. All *PaLAR3* genotypes had viable emblings after the first growing season in the nursery but the frequency varied depending on the *PaLAR3* genotype (Figure 3). Mean viability percentages of different *PaLAR3* genotypes for the four SE-families were 67.7, 56.7, and 62.7 for *PaLAR3* AA, AB, and BB emblings (Figure 3A). The emblings derived from *PaLAR3B* heterozygotic SE lines had lower viability compared to *PaLAR3A* homozygotic emblings ($X^2 = 12.01$, df = 2, $p < 0.01$) but did not differ from *PaLAR3B* homozygotes (Figure 3A). Within different families, the mean viability percentages varied from 41.6% in E242 × E222 to 80.8% in E46 × E3222. When taking the genetic background into consideration, we found that *PaLAR3* allele composition affected viability within three families (Figure 3B). In families E242 × E222 and E9 × E3231, the *PaLAR3* heterozygote viability was ca 32% lower compared to *PaLAR3A* homozygotes (in E242 × E222, the viability was 61.7% and 41.6%, and in E9 × E3231, 62.0% and 42.1% for AA and AB genotypes, respectively). Among the SE-progeny of E46 × E3222, the *PaLAR3B* homozygotes had 23.2% lower viability compared to their heterozygotes (62.0% vs. 80.7%).

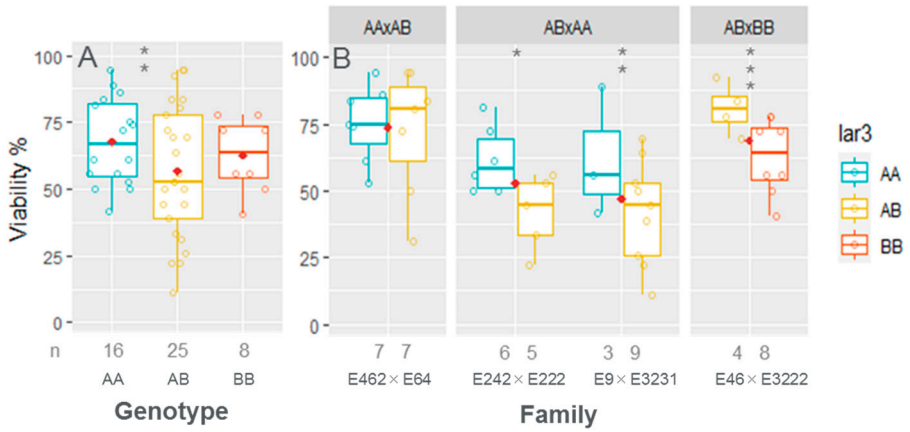

Figure 3. Embling viability (%) of different *PaLAR3* genotypes (**A**) of four Norway spruce full-sib SE-families (**B**) in the end of first growing season with the number of SE lines given for each allelic combination (*n*). Asterisks denote differences between genotypes (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4. Discussion

The present study shows, for the first time, that the root rot resistance allele *PaLAR3B* is successfully carried through plant regeneration by somatic embryogenesis (SE) in Norway spruce. In addition, we developed a simple single-tube genotyping assay for insertion/deletion in the *PaLAR3* alleles that was applied in the selection of plant material in tissue culture processes and can be used in conventional breeding and selection processes. The study reveals that 47.5% of the Finnish plus-trees recently used for SE-plant production carry the resistance allele, and that the allele frequency of *PaLAR3B* is 0.25 in the trees used to generate crosses for SE-initiations during 2011–2019. The estimated allele frequency is in line with previous studies, where the average *PaLAR3B* allele frequency was reported to be 0.17 in the Finnish Norway spruce populations (Punkaharju 0.18, Tuusula 0.16) [6] and showed the potential to deploy *PaLAR3B* through selection of the appropriate clonal material.

The SE-initiation percentages varied between years and full-sib families representing varying *PaLAR3* genotypes as parents, and when these factors were included in analyses, family explained the variation in SE-initiation success while *PaLAR3* genotype did not have effect on initiation. Large variations in the SE-initiation success among families is well-known in Norway spruce, e.g., from 30% to over 90% [30]. The genotyping further showed that in most analyzed families of SE lines, the allele delivery ratio followed the Mendelian principle of inheritance.

The effect of the genotype at the *PaLAR3* locus on the potential to form mature embryos differed between families, and in the most of families, there were no differences in E/gFW. It is well known that there is a large variation in embryo production capacity, not only between the Norway spruce genotypes but also on the family level [30,31]. Thus, the observed differences in potential to form mature embryos in these families are likely related to other components of the genetic background rather than the *PaLAR3* genotype.

All selected embryos, independent of their genotype at the *PaLAR3* locus, germinated in vitro and were transplanted to nursery. *PaLAR3* is known to be the most highly expressed *LAR* gene with rather consistent expression levels in a tissue panel from Norway spruce saplings [8]. Furthermore, in a previously published comparison of the transcriptome of normal and aberrant Norway spruce somatic embryo germinants, *PaLAR3* was not among the differentially expressed genes [32]. Together, these analyses suggest that *PaLAR3* and its activity do not play any major role during germination. The high in vitro germination rate is related to short duration of in vitro germination used for the present material and has been previously proved to give good results [20,30].

After the first growing season in the nursery, the viability of the emblings carrying the *PaLAR3B* resistance allele was lower, i.e., a lower frequency of plants remained in the nursery, in two families compared to the emblings without the allele. The observed 23.2% to

32.1% decrease in viability rate in the three families may be related to the overall genotypic differences between families shown to exist in Norway spruce [30], but may also be an indication of a trade-off between the higher transcriptional activity of *PaLAR3B* allele and viability during early growth of the emblings under nursery conditions. First, *PaLAR3B* homozygotes are known to have higher (+)-catechin content in bark [6]; second, *PaLAR3* is the most abundant *LAR* in Norway spruce and, therefore, perhaps also responsible for the bulk of catechin production [6,8]; third, higher catechin level in dormant buds is known to correlate with delayed bud flush in both Norway spruce and silver fir (*Abies alba*) [33]. Thus, it is possible that the higher activity of the *PaLAR3B* allele may affect the viability of the young emblings, for instance, by influencing metabolic fluxes and resource allocation in the embling during the first growth season. In a recent study performed with Norway spruce emblings, Puentes et al. [34] showed that the SE-propagation method is associated with increased protection against *Hylobius abietis* damage, and suggested that it may be because the propagation method induces genes involved in the tree's biotic defense, e.g., increasing the production of secondary compounds. Further studies with broader SE materials and conditions in the nursery are needed to clarify the effect of the *PaLAR3* allele on the viability of emblings after the first growth season.

There was also remarkable within-family variation in the nursery viability among the SE lines carrying the *PaLAR3B* allele, indicating the possibility to find SE lines having the *PaLAR3B* allele and also good embling viability in the nursery. This enables deployment of forest regeneration material carrying resistance traits. Reducing relatedness of propagated genotypes in commercial lots and having material with higher genetic diversity have been suggested to mitigate the risks associated with both depletion of genetic diversity and the success rate of SE-propagation [11], recognized as potential obstacles for the deployment of SE-material in practical forestry [35].

In future, the SE-material with known *PaLAR3* genotypes may be superior both for deployment and for further research regarding other resistance markers than *PaLAR3*. The clonal nature of the SE-material together with cryostorage for juvenility maintenance enables through testing of the same genotypes in different locations and conditions and, thus, selection of the best material for production of high-quality forest regeneration material including resistance traits. The deployment of marker-assisted selection in SE-pipeline may foster the production of more root rot-resistant forest regeneration material sooner than previously anticipated.

5. Conclusions

The present study demonstrates, for the first time, that the root rot resistance locus *PaLAR3B* is successfully delivered from elite Norway spruce parent trees to their SE-progeny through the SE-propagation method. This represents a major step towards a fast deployment of the allele in Nordic forests. Although the results show that the resistance allele may interfere with embling establishment in the nursery during the first growth season, it does not affect SE-initiation success or embryo production capacity.

Furthermore, the developed simple and efficient genotyping assay will aid in the selection of parent trees as well their SE-progeny with desired *PaLAR3* genotypes for both breeding and deployment of more resistant forest regeneration material.

Supplementary Materials: The following are available online at https://www.mdpi.com/1999-4907/12/2/193/s1, Table S1: Primers used for genotyping of *PaLAR3* alleles. Table S2: Numbers of SE-initiations (initiation attempts, i.e., explants) and successful initiations in each full-sib family parent *PaLAR3* crosstype. Table S3: Logistic regression model used for analyzing binary response in SE-initiations.

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Data Availability Statement: The data is available upon request to Jaanika Edesi (Jaanika.Edesi@luke.fi or Tuija Aronen (Tuija.Aronen@luke.fi).

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Conflicts of Interest: The authors declare no conflict of interest.

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