



Functional biotechnology

Interspecific somatic hybrids *Solanum villosum* (+) *S. tuberosum*, resistant to *Phytophthora infestans*



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ABSTRACT

The interspecific somatic hybrids 4x *S. villosum* (+) 2x *S. tuberosum* clone DG 81-68 (VT hybrids) were obtained and characterized molecularly and cytogenetically.

The morphology of fusion-derived plants was intermediate in relation to the parental species. The expected ploidy level of the regenerants was 6x for the VT hybrids, but the real ploidy of the hybrids varied, with some of them being euploids, and others – aneuploids. The hybridity of the regenerants was verified by random amplified polymorphic DNA (RAPD) analysis. Despite the variation in ploidy, the RAPD patterns of the hybrids were mostly uniform, suggesting similarity of the genotypes of the VT clones. Genomic *in situ* hybridization (GISH) analysis discriminated between the chromosomes of both parental genomes in VT somatic hybrids and also confirmed their hybridity. The resistance of VT somatic hybrids to *Phytophthora infestans* was evaluated and all of the hybrids proved to be highly resistant. In search of the mechanisms involved in resistance of the *Solanum* species to *P. infestans*, the biochemical reactions occurring early after elicitor treatment were studied. The production of reactive oxygen species (ROS), as one of the earliest reactions induced by pathogens or their elicitors, was examined in the resistant wild species *S. villosum*, susceptible *S. tuberosum* clone DG 81-68 and in the VT hybrid, resistant to *P. infestans*. After treatment of the leaves with elicitor, the relative increase in ROS production was higher in leaves of the susceptible potato clone than in the resistant plants of *S. villosum* and the somatic hybrid.

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Introduction

Phytophthora infestans (Mont.) de Bary, the pathogenic oomycete, causes late blight – the most destructive potato disease (Kamoun and Smart, 2005; Sarkar et al., 2011). The damage caused by *P. infestans* in agricultural crops is tremendous due to difficulties in controlling the spread of the disease. The use of chemicals targeted against *P. infestans* can provide some level of disease control. However, in a long term, the development of potato crops possessing durable genetic resistance, both by classical breeding and/or by genetic engineering methods, provides the best prospect

for effective economic and environmentally acceptable control of late blight disease.

There are different types of resistance to *P. infestans*, and the resistance level varies in potato cultivars as well as in numerous wild *Solanum* species. It is generally accepted that the race- or cultivar-specific plant resistance requires the presence of both a plant resistance (*R*) gene and matching pathogen avirulence (*Avr*) gene, whereas non-host and quantitative (partial, field) plant resistances are polygenically controlled (Keller et al., 2000; Kamoun and Smart, 2005; Kou and Wang, 2010). In the interaction between immune wild *Solanum* genotypes and *P. infestans*, the non-host type of resistance can be explained by the presence of a whole arsenal of *R* genes interacting with a number of *Avr* genes, whereas partial resistance could result from “weak” *R* gene–*Avr* gene interactions or gene-dosage effects. On the other hand, biochemical defense responses in host and non-host resistance are very similar, although the latter does not require an apparent *R/Avr* gene combination. In *Solanum/P. infestans* interactions, wild *Solanum* species often express non-host resistance, whereas cultivated potatoes represent mostly quantitative resistance manifested by various levels of disease reduction. Moreover, in cultivated potato, the presence

Abbreviations: *Avr* gene, avirulence gene; CF, culture filtrate; cvs, cultivars; DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; GISH, genomic *in situ* hybridization; HR, hypersensitive response; NBT, nitroblue tetrazolium; *R* gene, resistance gene; RAPD, random amplified polymorphic DNA; ROS, reactive oxygen species; *tbr*, *Solanum tuberosum*; *vll*, *Solanum villosum*; VT hybrids, somatic hybrids of 4x *S. villosum* (+) 2x *S. tuberosum* clone DG 81-68.

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of some unidentified *R* genes has not been excluded (Vleeshouwers et al., 2000). Studies by Tian et al. (2006) have indicated that different wild *Solanum* species, potato cultivars with various levels of resistance, and representative non-host species infected with *P. infestans*, displayed a hypersensitive response (HR). Polygenic resistance to *P. infestans* may be of great value in potato breeding since it seems to be the only durable resistance to this disease identified so far. Some *R* genes can also be more durable than others. The *RB* gene (also known as *Rpi-blb1*) of *Solanum bulbocastanum* recognizes a broad spectrum of *P. infestans* isolates and appears to be durable in the field (Helgeson et al., 1998; Song et al., 2003; Vleeshouwers et al., 2008).

The improvement of potato resistance to late blight disease can be achieved by introgression of the *R* genes from wild *Solanum* species by different biotechnological methods, including, among others, somatic hybridization (Orczyk et al., 2003). This method allows to create potato genotypes with enhanced resistance to various biotic and abiotic stresses, including resistance to *P. infestans* and *Globoderia pallida* from *S. berthaultii* (Serraf et al., 1991), resistance to potato virus Y (PVY) and *P. infestans* from *S. tarnii* (Thieme et al., 2008). In the latter, the obtained somatic hybrids were successfully backcrossed with cultivated potato (Thieme et al., 2008). Somatic hybridization has proven to be a valuable tool for producing pre-breeding material with increased genetic diversity and has unique potential to transfer simultaneously both nuclear and cytoplasmic genes (Zhou et al., 2001; Tiwari et al., 2010). Because of the existence of different kinds of crossing barriers between wild and cultivated *Solanum* species, protoplast fusion is a method which allows to omit most of them (Szczepakowa et al., 2010).

In our lab we have previously conducted successful fusion experiments between cultivated potato and several late blight-resistant wild *Solanum* species, such as *S. bulbocastanum* (Szczepakowa et al., 2003a), *S. nigrum* (Szczepakowa et al., 2003b), *S. pinnatisectum* (Szczepakowa et al., 2005) and *S. × michoacanum* (Szczepakowa et al., 2010). The number of somatic hybrids obtained varied for different fusion combinations, and the hybrid level of resistance against *P. infestans* was not always as high as that of the wild parent, being insufficient for breeding programs (Szczepakowa et al., 2010). In addition to improvement of potato resistance against *P. infestans*, our research interests concern the defense mechanisms in *Solanum* species in response to *P. infestans* (Polkowska-Kowalczyk et al., 2004, 2007, 2008, 2011). Our studies showed that the same early reactions to elicitor from *P. infestans* occurred both in resistant and susceptible *Solanum* species, although they differed in speed and intensity depending on the level of resistance of the species.

Studies of the mechanisms of plants' responses to pathogen attack are useful for elucidating the physiological and molecular bases of plant resistance and for plant breeding. Biochemical reports concerning polygenic resistance are limited. The diversity of potato genotypes complicates the understanding of biochemical reactions occurring in the infected plants. Moreover, phylogenetic analyses suggest that oomycetes form a unique group of eukaryotic plant pathogens that may display distinct genetic and biochemical mechanisms in interactions with plants (Kamoun et al., 1999; Kamoun and Smart, 2005). Experiments on various *Solanum* species expressing different types and levels of resistance against *P. infestans* may help to understand reactions induced by pathogen or pathogen-derived elicitors.

One of the earliest observable events of plants' defense mechanisms to biotic stresses is oxidative burst, an accelerated production of reactive oxygen species (ROS) (Halliwell, 2006; Marino et al., 2012). The generated ROS are considered to play a key role in the integration of diverse strategies, leading to disease resistance. ROS may act as signal transduction

agents for activating defense pathways and as mediators in HR.

Solanum villosum Mill. ($2n=4x=48$), a wild *Solanum* species, commonly known as red-fruit nightshade, is used by many African tribes because of its nutritive value. *S. villosum* is believed to have originated in Eurasia, and is sometimes considered to have a southern European origin. It is widespread, but absent in Central and South America, and in New Guinea. Scientific reports concerning *S. villosum* are scarce. Ojiewo et al. (2006) have developed two genotypes from wild-type tetraploid *S. villosum* ($2n=4x=48$). After application of colchicine to *S. villosum*, octoploid plants ($2n=8x=96$) were obtained. These plants had larger stomata, fewer but larger leaves, and were late flowering as compared to the wild-type tetraploids (Ojiewo et al., 2006; Masinde et al., 2007). An increase in ploidy level resulted in greater vigor and more intensive growth of $8x$ plants (Masinde et al., 2009). *S. villosum* is completely resistant to *P. infestans* since neither spreading lesions nor sporulation were observed on it in a number of experiments. Hence, this species may be used as a source of resistance for breeding potato (Colon et al., 1993; Lebecka, 2008).

In this paper, we present the molecular and cytogenetic characteristics of somatic hybrids obtained via polyethylene glycol-mediated chemical fusion performed in order to transfer resistance to *P. infestans* from the wild tetraploid *S. villosum* to a susceptible diploid clone of *S. tuberosum*. We present *S. villosum* (+)*S. tuberosum* (VT) somatic hybrids' morphology, ploidy, genome composition and resistance to *P. infestans*. We also report the generation of ROS during elicitor (culture filtrate of *P. infestans*, CF) treatment in both parental species and in the interspecific somatic hybrid, expressing different levels of resistance to *P. infestans*.

The detailed characteristics of VT somatic hybrids may help in further selection of the best clones for breeding tests. Moreover, comparative studies of the kinetics and intensity of ROS production may lead to a better understanding of the mechanisms involved in polygenic resistance.

Materials and methods

Plant material

The late blight-susceptible diploid *Solanum tuberosum* clone DG 81-68 ($2n=2x=24$) is a potato breeding line obtained in IHAR-PIB, Młochów Research Center, Poland. Seeds of the tetraploid wild species *Solanum villosum* Mill. ($2n=4x=48$), highly resistant to *Phytophthora infestans*, were provided by Warsaw Botanical Garden, Poland. The species origin is Birmingham, UK, and its accession no. is 4.

Axenic shoots of the parental species (received from IHAR-PIB, Młochów Research Center, Poland) and the generated interspecific somatic hybrids expressing different levels of resistance to the oomycete pathogen *P. infestans* were cultured *in vitro* as described previously (Polkowska-Kowalczyk et al., 2004). The plants grew under controlled conditions: day/light fluorescent lamp $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 16 h, day/night temperature of 22/18 °C.

The protoplasts isolated from leaves of the parental species were fused in the presence of polyethylene glycol according to Szczepakowa et al. (2003a,b). The regenerated somatic hybrids were propagated and grown *in vitro* on MS/2 hormone-free medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 0.6% agar.

Random amplified polymorphic DNA (RAPD) analysis

For verification of the hybridity, the RAPD analysis was used. The total genomic DNA was isolated from leaves of 3-week-old *in vitro*

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