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#Note to Syst Appl Microbiol#

Note: Development of a 16S rRNA-targeted fluorescence *in situ* hybridization probe for quantification of the ammonia-oxidizer *Nitrosotalea devanaterra* and its relatives

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Abstract

The *Thaumarchaeota* SAGMCG-1 group and, in particular, members of the genus *Nitrosotalea* have high occurrence in acidic soils, the rhizosphere, groundwater and oligotrophic lakes, and play a potential role in nitrogen cycling. In this study, the specific oligonucleotide fluorescence *in situ* hybridization probe SAG357 was designed for this *Thaumarchaeota* group based on the available 16S rRNA gene sequences in databases, and included the ammonia-oxidizing species *Nitrosotalea devanaterra*. Cell permeabilization for catalyzed reporter deposition fluorescence *in situ* detection and the hybridization conditions were optimized on enrichment cultures of the target species *N. devanaterra*, as well as the non-target ammonia-oxidizing archaeon *Nitrosopumilus maritimus*. Probe specificity was improved with a competitor oligonucleotide, and fluorescence intensity and cell visualization were enhanced by the design and application of two adjacent helpers. Probe performance was tested in soil samples along a pH gradient, and counting results matched the expected *in situ* distributions. Probe SAG357 and the CARD-FISH protocol developed in the present study will help to improve the current understanding of the ecology and physiology of *N. devanaterra* and its relatives in natural environments.

Introduction

Thaumarchaeota were initially named as Marine Group I *Archaea* and were considered part of the crenarchaeotal phylum according to the 16S ribosomal RNA gene phylogeny [17,23]. However, subsequent studies using comparative genomics allocated *Thaumarchaeota* to a separate and deep-branching phylum within the domain *Archaea* [13]. Recent meta-analyses have confirmed that members of the phylum *Thaumarchaeota* are highly diverse and are present in a wide variety of ecosystems, including marine, freshwater, soil, sediment, biofilm and hot springs [20,21]. The phylum contains not only ammonia-oxidizing archaea (AOA) but also environmental sequences representing microorganisms of unknown metabolism that are spread in different clades, such as *Nitrosopumilus maritimus*, *Nitrosoarchaeum limnia*, *Nitrosotalea devanaterra*, or *Nitrososphaera gargensis*, among others [30,33]. The discovery and cultivation of the chemolithotrophic, obligate acidophilic thaumarchaeal ammonia-oxidizing species *Nitrosotalea devanaterra* [24] confirmed that many members of the SAGMCG-1 group could be involved in nitrogen cycling. This group and, in particular, members of the genus *Nitrosotalea* have a high occurrence in oligotrophic freshwaters [6,7] and acidic soils [33]. Abundance distributions, recurrent appearance and significant correlation with nitrogen transformation have suggested a key role of SAGMCG-1 in the nitrogen cycling of oligotrophic alpine lakes [8,9,35].

In the last 25 years, great attention has been paid to the optimization of genetic tools for the rapid and accurate *in situ* estimation of microbial communities. Quantitative polymerase chain reaction (qPCR) has been largely used for quantification of microbial communities, and several optimized protocols are currently available and widely applied for the quantification of the 16S rRNA gene [19]. However, qPCR can introduce certain biases in quantification, mainly due to the loss of DNA during the different handling procedures [19]. *In situ* hybridization is a quantification methodology independent of nucleic acid extraction biases that is able to quantify accurately the number of cells, and can thus circumvent some of these reported limitations [32].

Fluorescence *in situ* hybridization (FISH) is an ideal method for the phylogenetic identification and counting of microorganisms in natural environments [2,3], and numerous fluorescently labeled specific oligonucleotide probes have been designed [4,16]. FISH has been successfully applied to many environmental samples, such as

sediments [31], roots [5], and soil [14], among others. However, the application of the FISH technique to environmental samples was hampered by weak signal intensity due to the low rRNA content of target cells, signal quenching, inhibited target site accessibility, and high levels of background fluorescence [4,10]. To overcome these limitations and to improve the detection of target cells, catalyzed reporter deposition [11] was coupled to fluorescence *in situ* hybridization (CARD-FISH) and successfully applied in oligotrophic environments where cells have low activity and low ribosome content [32].

In this study, the aim was to develop and optimize the application of a 16S rRNA-targeted oligonucleotide probe for the thaumarchaeal lineage SAGMCG-1, as well as optimize a CARD-FISH protocol for the *in situ* identification and enumeration of the ammonia-oxidizer *Nitrosotalea devanaterra* and its relatives.

Materials and Methods

Probe design and synthesis

Probe design was carried out with ARB [27], using publically available 16S rRNA gene sequences. Highly related sequences of several typical SAGMCG-1 sequences were searched in GenBank using blastn [1], and included additional environmental sequences when needed [9,35]. The 16S rRNA gene sequences were automatically aligned with the Nearest Alignment Space Termination (NAST) algorithm [18], clustered at a 97% identity threshold, and imported into the SILVA database [34] using "ARB parsimony (quick add marked)" with the filter "Positional variability by parsimony for Archaea". Neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) (all three embedded in ARB) and posterior probability trees were calculated for SAGMCG-1 group sequences. Probe, helper and competitor designs were undertaken in ARB, using a PT-server built from all sequences in the database. Analysis of the probe target and specificity was carried out with the programs TestProbe 3.0 from SILVA [34], and probeCheck and probeBase [26] from the Division of Microbial Ecology (DOME). Probe synthesis was undertaken by Biomers (Ulm, Germany).

Sample sources

For probe testing and optimization, AOA enrichment aerobic cultures and soil samples were used as provided by Scotland's Rural College (SRUC, Craibstone Estate, Aberdeen), and they were fixed in 4% paraformaldehyde (final concentration). *Nitrosotalea devanaterra* was grown in acidic (pH 4.5) aqueous medium, as described by Lehtovirta-Morley et al. [24], and *Nitrosopumilus maritimus* in HEPES-buffered synthetic medium (pH 7.6) [29]. Soil samples came from pH-manipulated experimental plots at the SRUC (Grid ref. NJ 867112) and were collected in August 2006. The soil plots had been maintained at 0.5 pH unit intervals in the range 4.5–7.5 for the past 40 years, by the addition of lime or aluminium sulphate [25]. Cells were extracted from the soil matrix using a Nycodenz® density gradient (Axis-Shield, Norway).

CARD-FISH protocol

For specific detection of *Thaumarchaeota* SAGMCG-1, the CARD-FISH protocol of Pernthaler et al. [32] was modified mostly at the cell permeabilization step (see Table S1 for details). Cells were permeabilized by incubation in freshly prepared lysozyme solution (10 mg mL^{-1} in 0.05 M EDTA, pH 8.0; 0.1 M Tris-HCl, pH 8.0) for 60 min at 37 °C, washed 3x in deionized water and 96% ethanol, and allowed to air dry. Subsequently, a second permeabilization step was carried out by incubation in freshly prepared achromopeptidase solution (60 U mL^{-1} in 5 M NaCl; 0.1 M Tris-HCl, pH 8.0) for 30 min at 37 °C. Hybridization was performed for 4 h at 35 °C on a rotary shaker, followed by a washing step for 10 min. CARD-amplification was undertaken for 15 min at 42 °C in the dark.

Cell counts

Cells were observed under a Zeiss Axio Imager fluorescence microscope with an X-Cite 120 lamp and an attached AxioCamMrm camera with AxioVision image acquisition PC software (Zeiss, Jena, Germany). A set of optical filters suitable for DAPI (Zeiss filter set #01: BP365/12, FT396, LP397), Alexa-Fluor 488 and Alexa Fluor 594 (Zeiss filter

sets #09: BP450-490, FT510, LP515, or set #24: DBP485/20, DFT500/600, BP515-540, LP610) were used. The ImageJ program (https://imagej.nih.gov/ij/) was used to calculate the efficiency of helpers by measuring the intensity of gray values under helper presence/absence for the same exposure time.

Results and Discussion

Defining the SAGMCG-1 clade

A total of 257 sequences were used for the phylogenetic tree and the design of specific probes. Phylogenetic analysis confirmed SAGMCG-1 as a monophyletic clade with very high bootstrap support (100%) that was separated consistently from Marine Group I (MG1). SAGMCG-1 was formed by two subclusters originating from different environmental sources, one of which was closely related to *Nitrosotalea devanaterra* (Fig. 1).

Probe design, competitors and helpers

Potential probe target regions for the SAGMCG-1 clade were positions 357-374, 362-378 and 530-547, based on *Escherichia coli* 16S rRNA numbering. Accordingly, three different potential probes were chosen for further investigation: SAG357, SAG362 and SAG530 that had different coverages in the dataset (90-96%) and a few unspecific matches (4-10%) mostly in the MG1 clade (Table 1). In order to solve the problem with the non-target group (MG1), competitors were designed [28]. Although probes SAG530 and SAG362 initially showed a slightly better specificity, the use of competitors resulted in a better performance for probe SAG357. Only one competitor was required for SAG357, since the coverage of the single mismatch with the competitor was enough to restrict hybridization of the probe with non-target organisms. Conversely, for SAG530 and SAG362, three and two competitors were necessary, respectively (Table 1). Thus, and since SAG362 and SAG530 did not yield a fluorescent signal for *Nitrosotalea devanaterra* cells in preliminary tests, the optimization of probe SAG357 was chosen, and helper oligonucleotides [22] located directly adjacent on both ends of the probe were subsequently designed (Table 1) to improve probe binding.

Cell permeabilization

Horse radish peroxidase, the label required for CARD-FISH probes, is too large (~40 kDa) to diffuse freely into cells [12]. Therefore, cells must first be permeabilized by partially degrading their cell walls. Enzymatic treatments using lysozyme [15], achromopeptidase, proteinase K, and pseudomurein endopeptidase are often employed for permeabilization. Sekar et al. [36] introduced achromopeptidase treatment following lysozyme treatment for permeabilization of *Actinobacteria* and, therefore, a combination of lysozyme and achromopeptidase was also optimized and applied to SAGMCG-1 (see details in Table S1). Achromopeptidase hydrolyzes lysyl peptide bonds [37], and lysozyme treatment probably improves the accessibility of achromopeptidase to the bonds.

Optimization of probe SAG357 and testing in complex soil samples

Hybridization conditions were optimized for *Nitrosotalea devanaterra* cells at 35 ºC in a 20-80% gradient of denaturant formamide, at 5% linear increments (v/v). As expected, increasing the formamide resulted in a sigmoid-like loss in cell detection efficiency starting at 45% formamide, and the non-target *Nitrosopumilus maritimus* cells were always below detection limits (Fig. 2). In addition, the fluorescence intensity conferred by probe SAG357 on *N. devanaterra* increased by one order of magnitude in the presence of helpers (Supplementary Fig. S1). A good agreement was observed for double hybridizations of SAG357 with the general archaeal probe ARCH915 (Fig. 3, panels A and B). The SAG357 probe used in complex soil samples (Fig. 3, panel C) showed a significant negative correlation ($r=-0.84$, $P<0.001$) between soil pH and cell concentration, with maximum abundances found at pH 4.5 and 5.0 (Fig. 4), as expected, which was in agreement with the previous findings for *N. devanaterra* [24].

Overall, probe SAG357 and the CARD-FISH protocol developed in the present study offers new possibilities for improving the understanding of the ecology and physiology of *N. devanaterra* and its relatives in natural environments.

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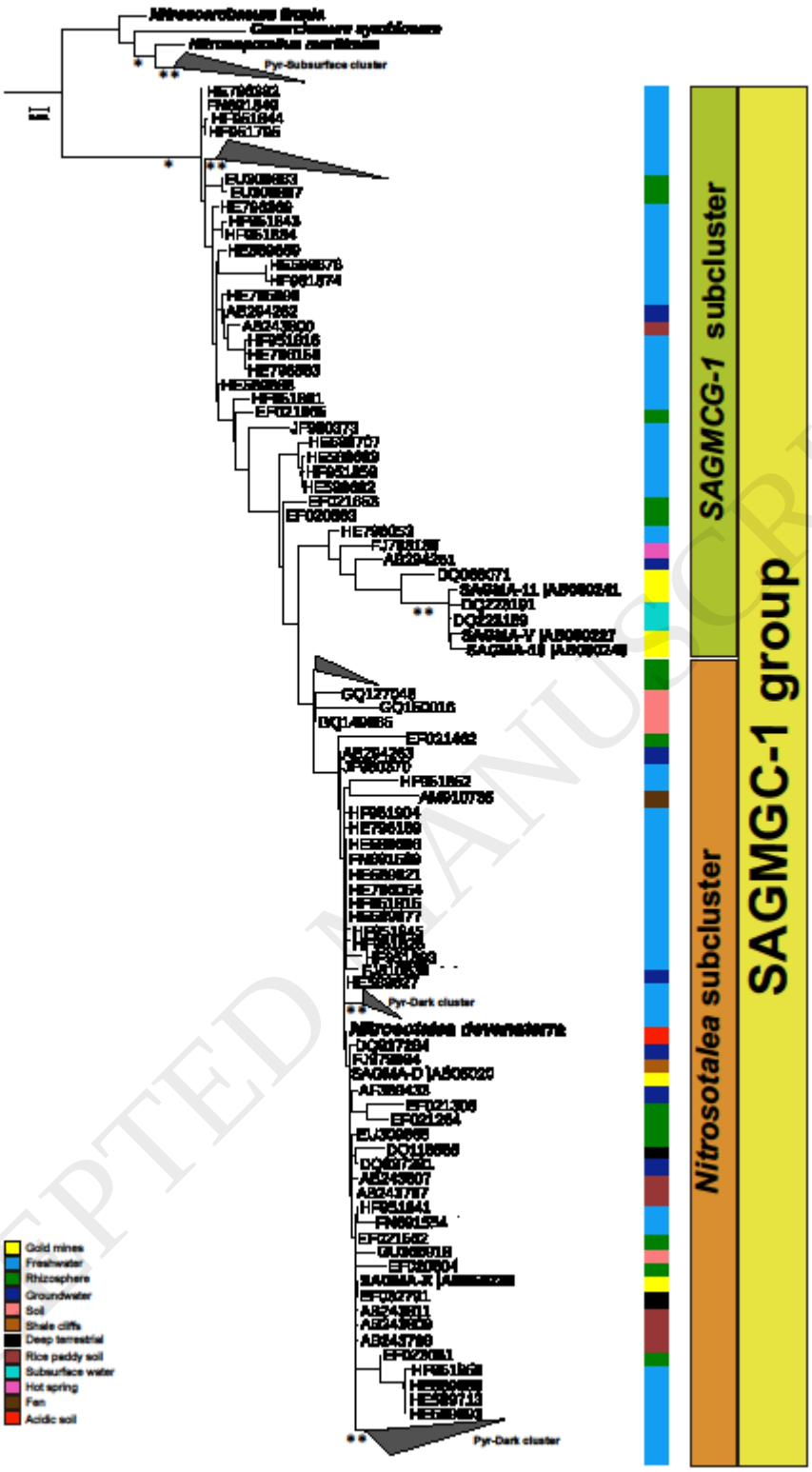
Figure Legends

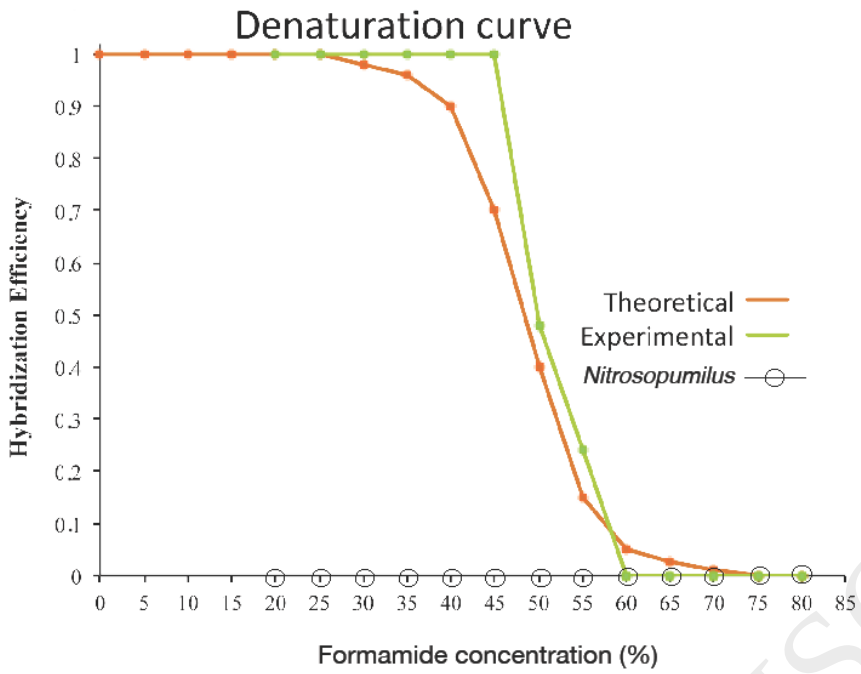
Fig. 1. SAGMCG-1 maximum likelihood (ML) phylogenetic tree with environmental features highlighted. Clustering is according to Restrepo-Ortiz et al. [35]. Bootstrap values: *0.88-0.94; **0.95 or higher. Scale bar: 0.01 mutation per nucleotide position.

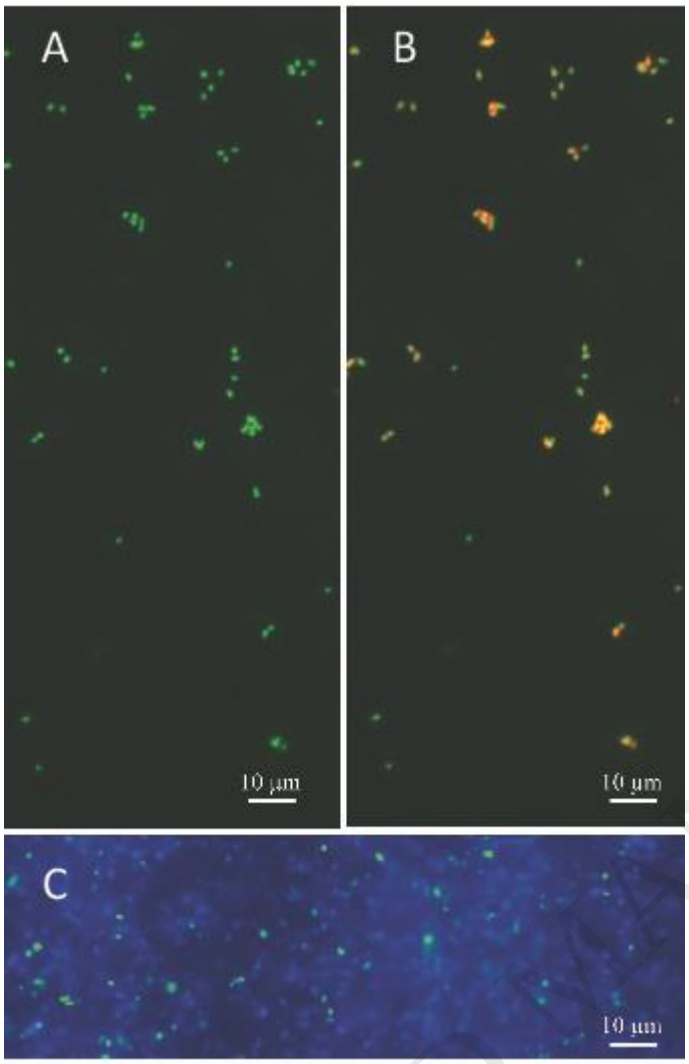
Fig. 2. Graphical display of experimental vs. theoretical formamide dissociation profiles for the SAG357 probe using strain *Nitrosotalea devanaterra* at increasing formamide concentrations and 35 ºC hybridization temperature. Optimal concentration was established at 45% formamide. The non-target *Nitrosopumilus maritimus* was always below detection limits.

Fig. 3. CARD-FISH SAG357 probe detection of *Nitrosotalea devanaterra* from enrichment cultures and environmental soil samples. Panel A: Hybridization in a *N. devanaterra* enrichment culture with SAG357-Alexa 488 (green) and (Panel B) double hybridization with ARCH915-Alexa 594 (red). Panel C: SAG357 labeling of *Thaumarchaeota* SAGMCG-1 in a pH 4.5 soil sample (pH-manipulated experimental soils, SRUC, Aberdeen).

Fig. 4. *In situ* quantification of *Nitrosotalea devanaterra* cells with probe SAG357 (45% FA, 35 ºC) in soil samples with increasing pH (pH-manipulated experimental soils, SRUC, Aberdeen).







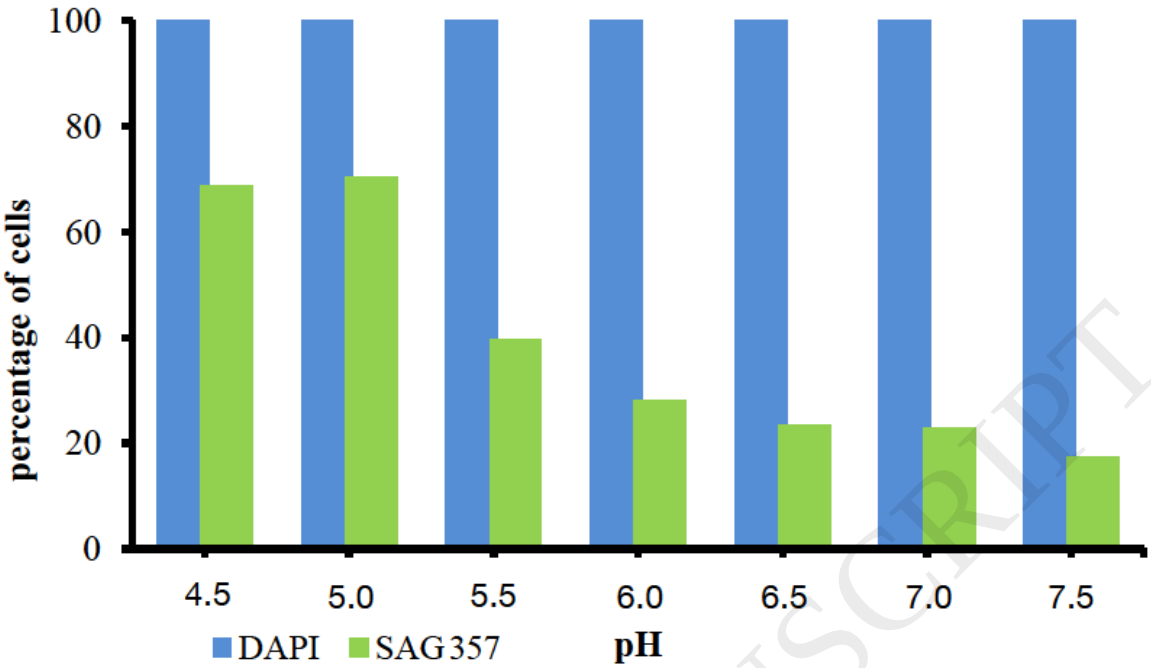


Table 1. CARD-FISH probes, competitors (*c*), and helpers (*h*) specific for targeting SAGMCG-1 designed in this study. The mismatches of the competitor with the target sequence are shown underlined in italics. Successful probe SAG357 with the corresponding helpers and competitor are in bold.

Probe	**Sequence**	**Position**	**GC%**	**Length (nt)**	**% TARGET in database**	
					SAGMCG-1	**Other**
SAG357	**5'-TTGCTAAGGTTTCTCGCC-3'**	357-374	50	18	90.1	9.9
***c*SAG357**	**5'-TTGC*A*AAGGTTTCTCGCC-3'**					
***h*SAG357_1**	**5'-TGCTGCGCCCCATAGGGCCTC-3'**					
***h*SAG357_2**	**3'-ACCYAGTCGTGCTTTCGCACA-5'**					
SAG362	5'-CACATTGCTAAGGTTTC-3'	362-378	41	17	96.2	3.8
*c*SAG362_1	5'-CACATTGC*G*AAGGTTTC-3'					
*c*SAG362_2	5'-CACATTGC*A*AAGGTTTC-3'					
SAG530	5'-TCGAGGTGCTGGTATTAC-3'	530-547	50	18	93.2	6.8
*c*SAG530_1	5'-TCGAGGTGCTGGT*T*TTAC-3'					
*c*SAG530_2	5'-TCG*G*GGTGCTGGTATTAC-3'					
*c*SAG530_3	5'-TCGAGG*A*GCTGGTATTAC-3'					