Reference genes for studying gene expression by Real-Time Quantitative RT-PCR in *Arabidopsis thaliana* exposed to increased concentrations of Cadmium and Copper

Tony Remans, Dennis Mathijsen, Karen Smeets, Kelly Opdenakker, Jaco Vangronsveld, Ann Cuypers
Environmental Biology, Hasselt University, Campus Diepenbeek, Agoralaan Building D, 3590 Diepenbeek, Belgium

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CENTRUM VOOR MILIEUKUNDE

CADMIUM and COPPER CAN CAUSE OXIDATIVE STRESS IN PLANTS

Plants need essential micro-elements to grow, some of which are metals (e.g. Copper, Cu). These elements can become phytotoxic at higher concentrations. Some metals are non-essential trace elements (e.g. Cadmium, Cd). From certain expposure levels, these non-essential trace elements are toxic for plants. Metal toxicity can cause a disruption of physiological processes, such as photosynthesis, transpiration,… which can lead to chlorosis, necrosis, reduced growth…

Even at low exposures to trace elements, when no obvious changes to the plant can be detected visually, a number of biological and molecular changes are taking place. Several studies have observed that the exposure of plants to toxic concentrations of metals provokes oxidative stress. Oxidative stress occurs when the balance between oxidants (such as reactive oxygen species, ROS), and antioxidants (which include certain enzymes and metabolites), is shifted towards the oxidants.

REFERENCE GENES FOR STUDYING *ARABIDOPSIS* GENE EXPRESSION UNDER Cd AND Cu EXPOSURE

In our studies of *Arabidopsis thaliana* exposed to Cd or Cu, we quantify gene expression of anti-oxidative defence genes (superoxidedismutases, catalases, peroxidases) and genes that can cause oxidative stress (NADPH oxidases, lipoxygenases).
We would like to compare the responses of these genes :
- in two tissues: roots and shoots
- to two metals: Cu and Cd
- at relatively high and low concentrations: Cu 0.5 μM and 2 μM; Cd 2 μM and 10 μM

Previously, especially when studying exposure to increased Cu concentrations, we found that some widely used reference genes for relative quantification were not stably expressed between control and different Cu treatments (e.g. Actin2).
In order to determine more suitable reference genes, we tested a series of genes for *Arabidopsis* as proposed by Czechowski *et al*, and used the geNorm algorithm (Vandesompele *et al*) to identify within this set of genes the ones that are the most stably expressed when studying the effects of exposure to Cu and Cd.

EXPERIMENT

Organism:
Arabidopsis thaliana, ecotype Columbio-0
Organs:
roots and shoots
Treatments:
relatively high Cu exposure (2 μM)
relatively low Cu exposure (0.5 μM)
relatively high Cd exposure (10 μM)
relatively low Cd exposure (2 μM)
no exposure (control treatment)

Figure 1: Arabidopsis hydroponic growth

Table 1: The tested genes have various expression levels relative to each other. The expression for each gene is very similar between roots and leaves.

GENE		LEAVES		ROOTS		D (L-R)
		average Ct	stdev	average Ct	stdev	
At5g25760	UBC	23.66	0.77	23.23	1.22	0.43
At5g15710	F-Box protein	26.34	0.64	25.77	0.69	0.57
At5g08290	mitosis protein YLS8	20.57	0.41	19.70	0.58	0.87
At5g55840	PPR gene	29.53	1.61	29.94	1.34	-0.41
At3g18780	Actin2	23.03	0.66	21.49	1.21	1.53
At2g28390	SAND family	24.43	0.57	23.94	1.15	0.50
At4g26410	expressed	23.60	0.79	22.85	0.91	0.75
At4g34270	TIP41-like	23.98	1.62	23.03	1.63	0.95

DETERMINATION OF STABLY EXPRESSED GENES

The expression ratio of two ideal reference genes is identical in all samples, regardless the treatment or organ. For candidate reference genes, these ratios are not identical, and based on the pairwise variation between the candidate reference gene expression in all organs and between all treatments, the geNorm algoritm (Vandesompele *et al.*) calculates a measure M, that reflects the expression stability of a candidate reference gene (lower M is more stable).
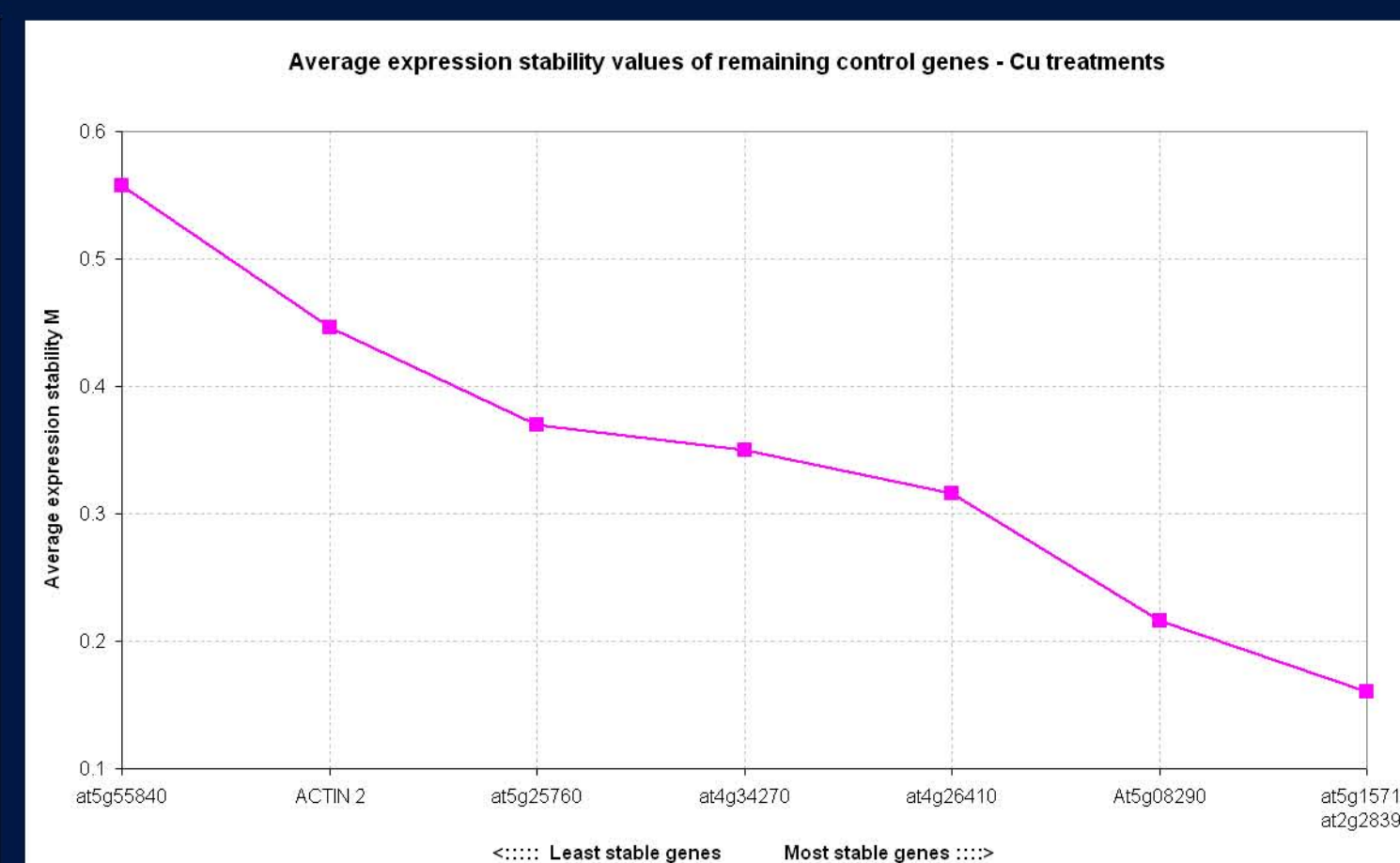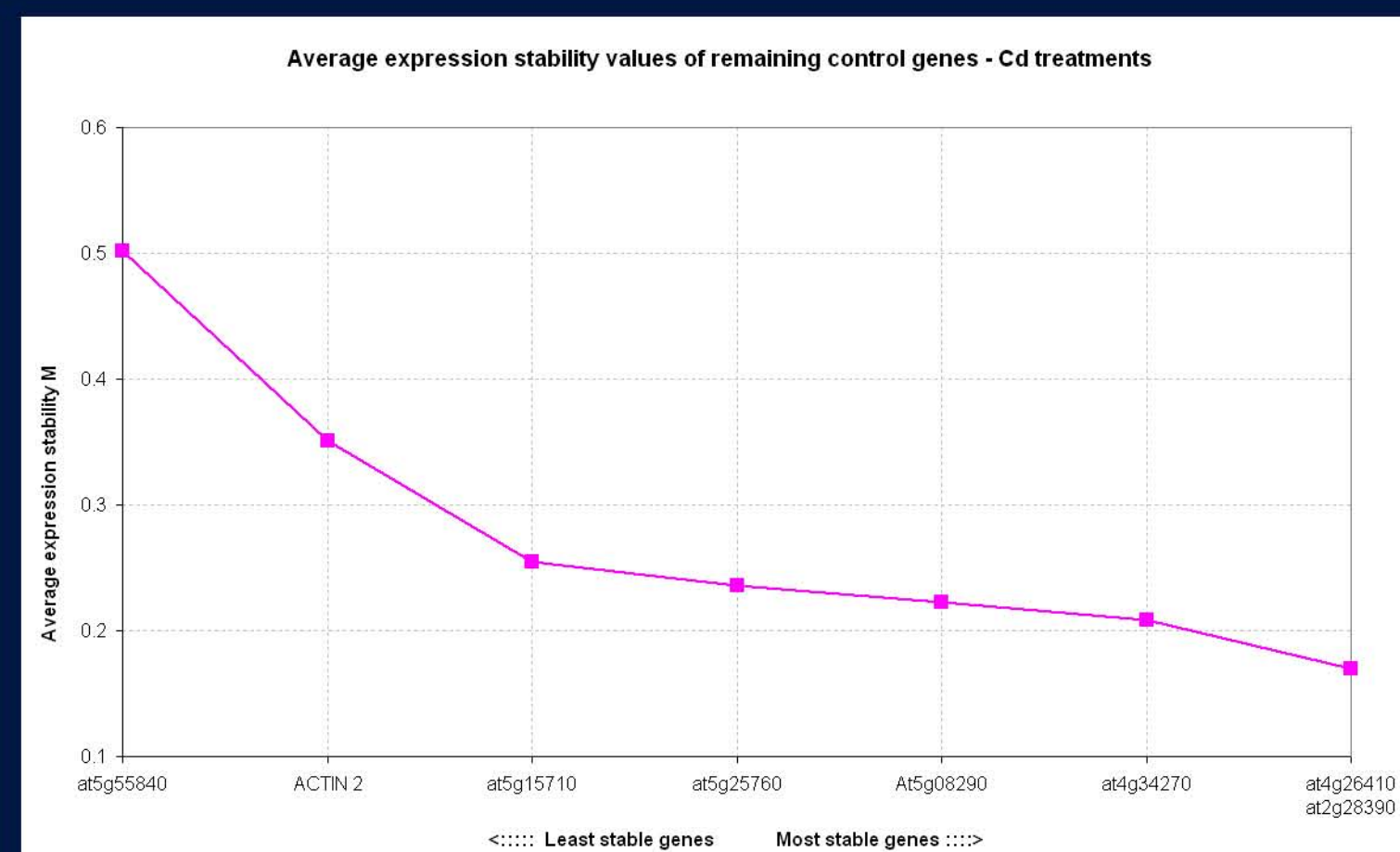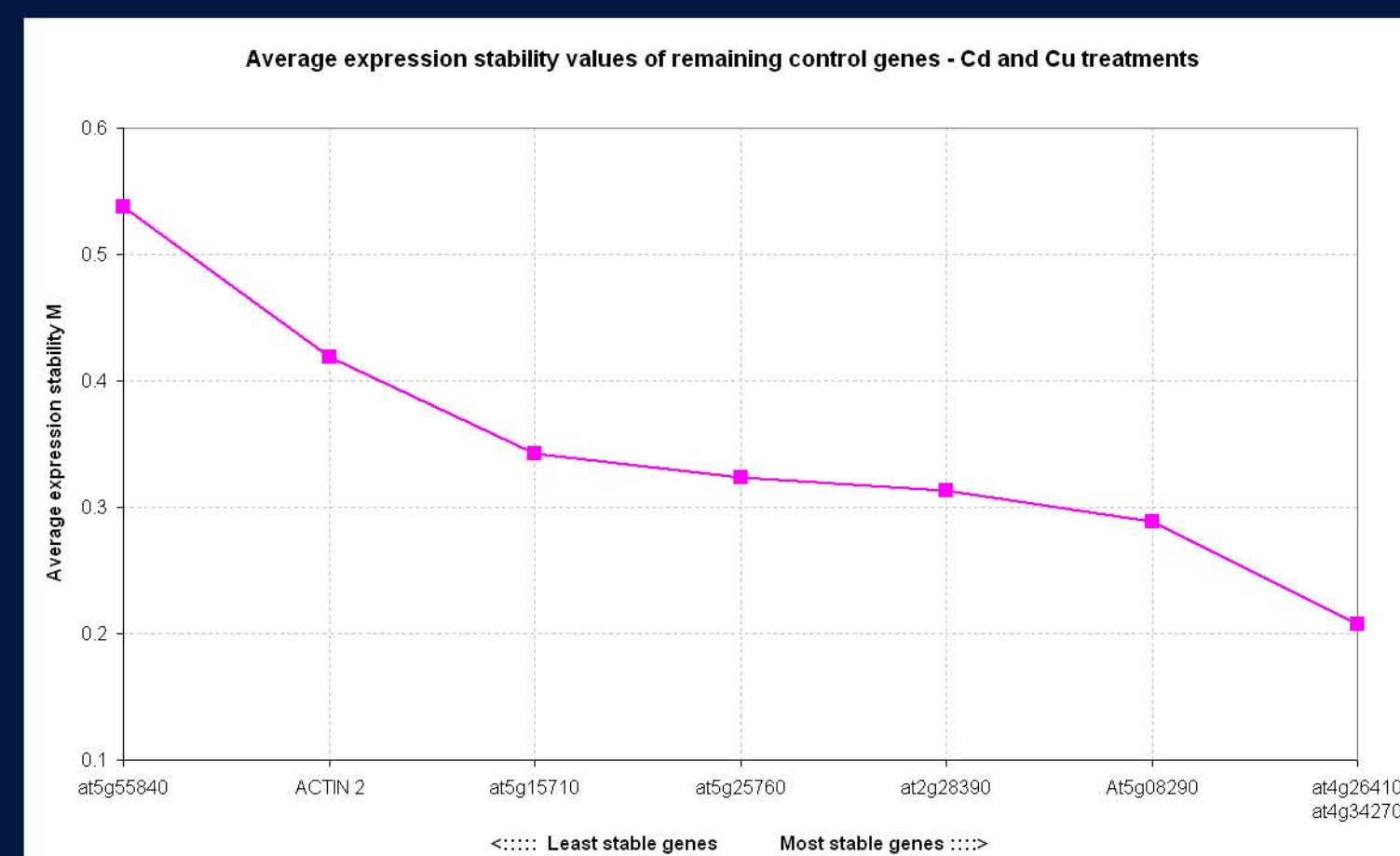

Figure 2: Stepwise exclusion of the gene with the highest M-value resulted in two housekeeping genes that have the most stable expression. We ranked the candidate reference genes when both Cd and Cu treatments were considered, and when Cd and Cu treatments were considered seperately. When considering the Cd treatments only, the M value of the tested genes was generally lower than when considering the Cu treatments only (more stable expression).
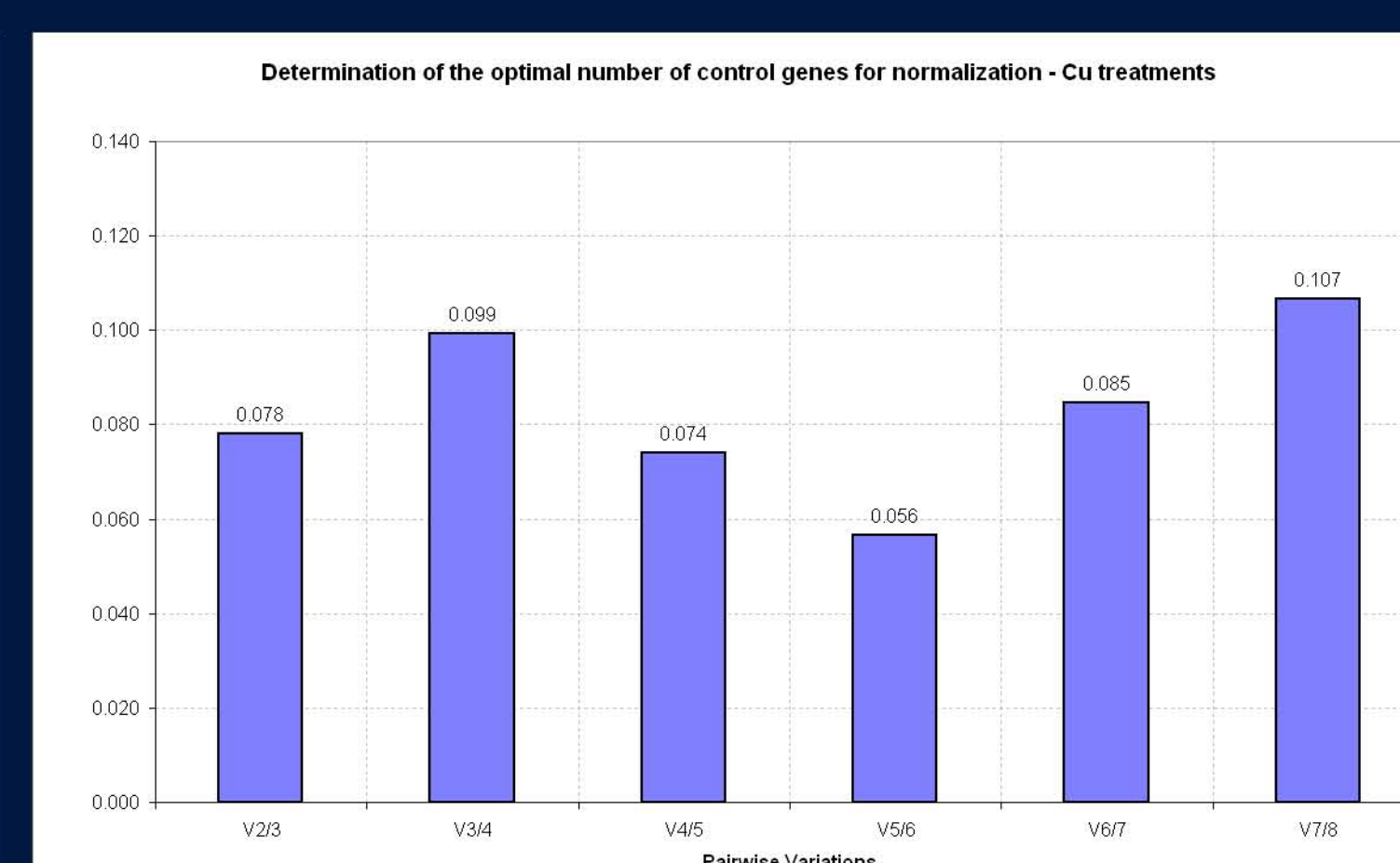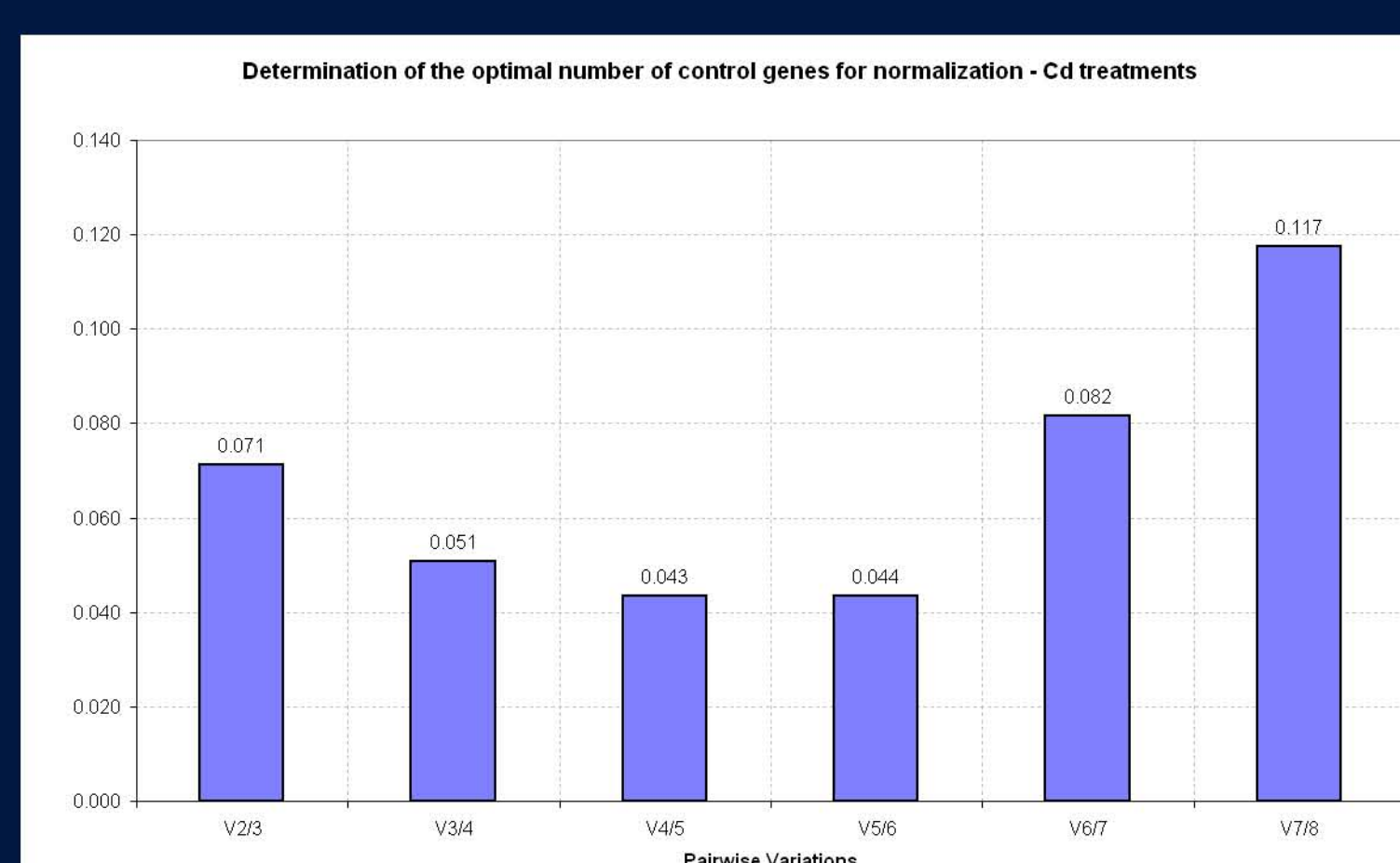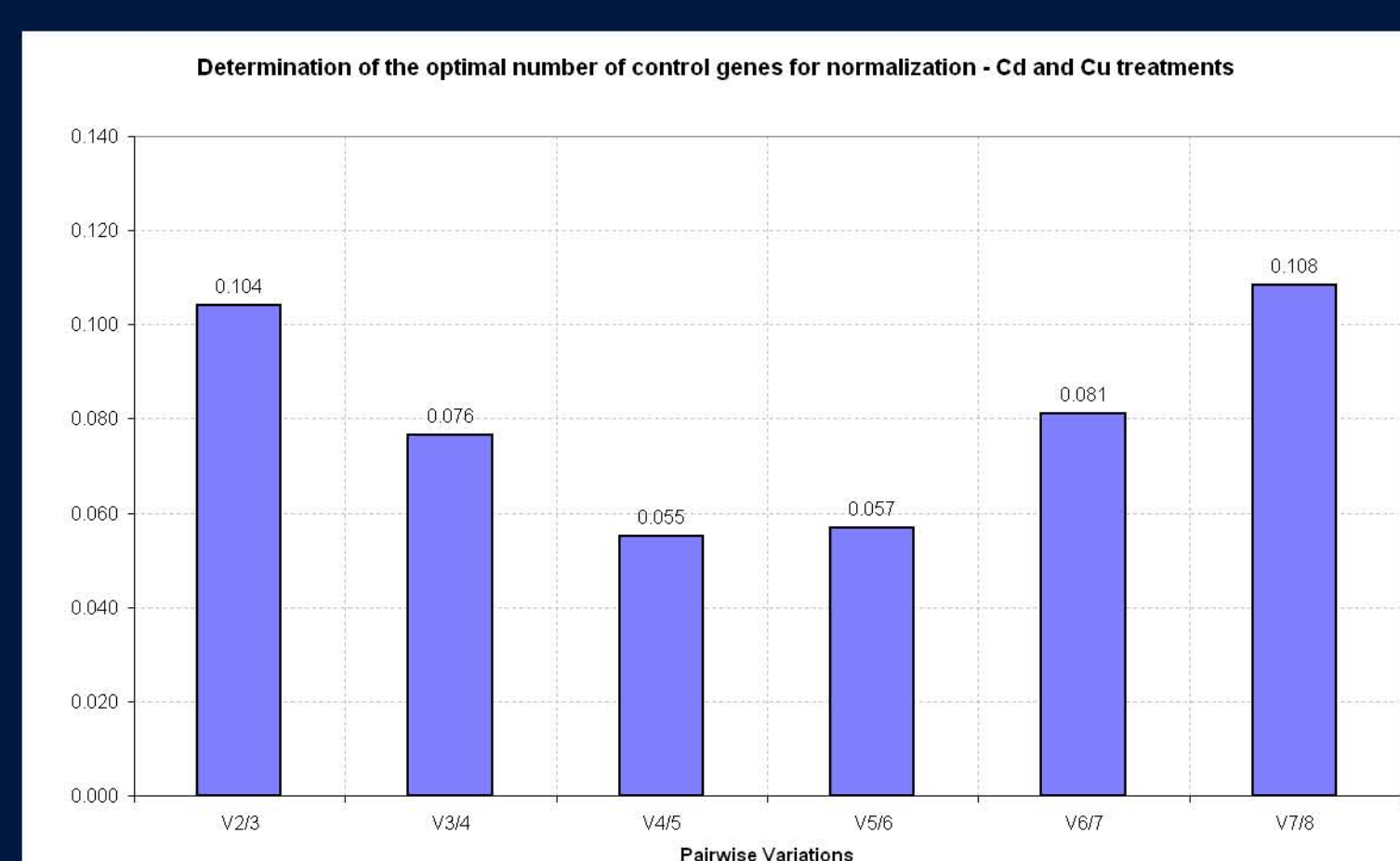

Figure 3: Determination of the optimal number of control genes for normalization. We will use the two most stably expressed genes, from which a normalisation factor is calculated as the geometric average of the expression for each sample. The pairwise variation measure V, which measures the effect of adding further reference genes on the normalisation factor, is relatively low already for adding a third gene (V2/3 <0.15).

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. Genome-wide identification and testing of superior reference genes for transcript normalisation in Arabidopsis. Plant Physiology 139 p.5-17 (2005).
Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 18;3(7):RESEARCH0034 (2002).